SUNFLOWER RhoGAP, LOX, ADH, AND SCIP-1 POLYNUCLEOTIDES AND METHODS OF USE

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/201,837, filed on May 3, 2000 and U.S. Provisional Application No. 60/166,128, filed on November 18, 1999.

FIELD OF THE INVENTION

The invention relates to the field of the genetic manipulation of plants, particularly the modulation of gene activity and development in plants and increased disease resistance.

BACKGROUND OF THE INVENTION

Disease in plants is caused by biotic and abiotic causes. Biotic causes include fungi, viruses, bacteria, and nematodes. An example of the importance of plant disease is illustrated by phytopathogenic fungi, which cause significant annual crop yield losses as well as devastating epidemics. Plant disease outbreaks have resulted in catastrophic crop failures that have triggered famines and caused major social change. Pathogenic fungi attack all of the approximately 300,000 species of flowering plants, however, a single plant species can be host to only a few fungal species, and similarly, most fungi usually have a limited host range. Generally, the best strategy for plant disease control is to use resistant cultivars selected or developed by plant breeders for this purpose. However, the potential for serious crop disease epidemics persists today, as evidenced by outbreaks of the Victoria blight of oats and southern corn leaf blight. Molecular methods of crop protection have the potential to implement novel mechanisms for disease resistance and can also be implemented more quickly than traditional breeding methods. Accordingly,

molecular methods are needed to supplement traditional breeding methods to protect plants from pathogen attack.

A host of cellular processes enable plants to defend themselves against disease caused by pathogenic agents. These defense mechanisms are activated by initial pathogen infection in a process known as elicitation. In elicitation, the host plant recognizes a pathogen-derived compound known as an elicitor; the plant then activates disease gene expression to limit further spread of the invading microorganism. It is generally believed that to overcome these plant defense mechanisms, plant pathogens must find a way to suppress elicitation as well as to overcome more physically-based barriers to infection, such as reinforcement and/or rearrangement of the actin filament networks near the cell's plasma membrane.

Thus, the present invention solves needs for enhancement of the plant's defensive elicitation response via a molecularly based mechanism that can be quickly incorporated into commercial crops.

SUMMARY OF THE INVENTION

The present invention provides nucleotide sequences that may find use in modulating development, developmental pathways, and the plant pathogen defense system. Particularly, the nucleotide and amino acid sequences for a sunflower rhoGTPase-Activating Protein (rhoGAP), Lipoxygenase (LOX), Alcohol Dehydrogenase (ADH), and *Sclerotinia*-Inducible Protein-1 (SCIP-1) are provided.

In particular, the methods and compositions can be used to modulate plant development. More specifically, methods and compositions of the invention may be used for enhancing resistance to plant pathogens including fungal pathogens, plant viruses, and the like. The method involves stably transforming a plant with a nucleotide sequence capable of modulating the plant pathogen defense system operably linked with a promoter capable of driving expression of a gene in a plant cell. The disease resistance genes of the present invention additionally find use in manipulating these processes in transformed plants and plant cells.

Transformed plants, plant cells, and seeds, as well as methods for making such plants, plant cells, and seeds are additionally provided. It is recognized that a variety of

promoters will be useful in the invention, the choice of which will depend in part upon the desired level of expression of the disclosed nucleotide sequences. It is recognized that the levels of expression can be controlled to modulate the levels of expression in the plant cell.

Methods and compositions for regulating gene expression in a plant are also provided. Novel nucleotide sequences for inducible plant promoters derived from the LOX and SCIP-1 genes are provided. The methods comprise transforming a plant with a nucleotide sequence of interest operably linked to the LOX or SCIP-1 promoters. Exposure of the transformed plant to a stimulus activates, within the exposed tissue of the plant, transcription of the nucleotide sequence of interest.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 schematically illustrates an expression vector containing the ubiquitin promoter operably linked the rhoGAP, LOX, ADH, and SCIP-1 nucleotide sequences.

Figure 2 schematically illustrates an expression vector used for plant transformation containing the LOX or SCIP-1 promoter nucleotide sequences operably linked to the nucleotide sequences encoding the GUS reporter protein.

DETAILED DESCRIPTION OF THE INVENTION

Overview

The present invention provides, inter alia, compositions and methods for modulating the total level of proteins of the present invention and/or altering their ratios in a plant. By "modulation" is intended an increase or a decrease in a particular character, quality, substance, or response.

The compositions comprise sunflower nucleic acid and amino acid sequences. Particularly, the nucleotide and amino acid sequence for a sunflower rhoGAP (SEQ ID NOS: 1 and 2), LOX (SEQ ID NOS: 3 and 4), ADH (SEQ ID NOS: 6 and 7) and SCIP-1 (SEQ ID NOS: 8 and 9) are provided. As discussed in more detail below, the sequences of the invention are involved in many basic biochemical pathways that regulate plant growth, development, and pathogen resistance. Methods are provided for the expression of these sequences in a host plant to modulate plant development, developmental

pathways, and defense responses. The method involves stably transforming a plant with a nucleotide sequence capable of modulating the plant pathogen defense system operably linked with a promoter capable of driving expression of the nucleotide sequence in a plant cell.

Also provided are LOX and SCIP-1 promoter sequences set forth in SEQ ID NO: 5 and SEQ ID NO: 10, respectively. Methods are provided for the regulated expression of a nucleotide sequence of interest that is operably linked to the LOX or SCIP-1 promoter sequences disclosed herein. Nucleotide sequences operably linked to the LOX or SCIP-1 promoters are transformed into a plant cell. Exposure of the transformed plant to a stimulus, induces transcriptional activation of the nucleotide sequences operably linked to the LOX or SCIP-1 promoters.

rhoGTPase-activating protein (rhoGAP)

A nucleic acid sequence encoding a rhoGAP polypeptide from sunflower is provided. The rhoGAP sequence shares homology to the conserved rhoGAP genes from humans. The sunflower rhoGAP amino acid sequence shares about 40% homology with the human p50rhoGAP sequence and about 80% homology with an *Arabidopsis* hypothetical 23.6 kDa protein.

RhoGAPs are a central part of an evolutionarily conserved regulatory system that are involved in cell growth and differentiation. Thus the sequence of the invention finds use in controlling or modulating cell division, differentiation, development, as well as the defense response. Transformed plants can be obtained having altered metabolic states with respect to cell division and cellular processes as well as development and defense response; hence, the methods and compositions may find uses in affecting or studying differentiation.

RhoGAP proteins have been shown to interact with rho members of the ras superfamily. Ras oncogenes were initially found to play an important role in human cancers and have since been shown to play important roles in regulation of cell growth and differentiation. Further, the rhoGAP proteins affect the activity of rhoGTPases (also called rho proteins), which act as molecular switches to regulate affected processes. The rho family of "G proteins" have a GTP-bound form and a GDP-bound form; the relative

amount of the GDP-bound form is increased by GTPase activating proteins, or GAPs, which stimulate the intrinsic GTPase activity of the rho proteins.

Processes affected by GAPs include the transduction of hormone signals across cell plasma membranes and the regulation of intracellular transport pathways. For example, rhoGTP-binding proteins have been shown to control signal transduction pathways connecting the activation of actin polymerization to activation of cellular growth factor receptors. Hence, the compositions and methods of the invention find use in the activation or modulation of the cellular actin cytoskeleton. Although there is a great deal of conservation among members of the rhoGAP family, there are a large number of different proteins that contain the rhoGAP domain, and many of these proteins are large and multifunctional. Thus, the rhoGAP genes and/or proteins may contain different elements or motifs or sequence patterns that modulate or affect the activity, subcellular localization, and/or target of the rhoGAP protein. Such elements, motifs, or sequence patterns may be useful in engineering novel enzymes for reducing or enhancing gene expression in particular tissues.

RhoGAP proteins activate rho genes and the related rac genes, which both stimulate actin polymerization. The rho proteins in mammalian systems have been shown to regulate the formation of multi-molecular complexes that are associated with polymerized actin located at the plasma membrane of the cell. Such complexes include actin stress fibers and focal adhesions in fibroblasts as well as the actin-driven phenomenon called membrane ruffling, which is exhibited by many cell types in response to extracellular stimuli. Rho proteins have also been shown to play roles in epithelial cell migration in response to wounding. Expression of the sequences of the invention can be used to modulate or regulate the expression of corresponding GTP-binding proteins, i.e., rho, rac, etc. Hence, the compositions and methods of the invention find use in the activation or modulation of the cellular actin cytoskeleton and other actin-based structures and actin-related processes.

The RhoGAP gene of the present invention additionally finds use in enhancing the plant pathogen defense system. Early plant-cell defense responses include the rearrangement of the cellular actin cytoskeleton to protect the cell from attack. RhoGAP genes are involved in cellular signaling cascades such as the oxidative burst that

comprises part of the early defense response in plants. Hence, the compositions and methods of the invention can be used for enhancing resistance to plant pathogens including fungal pathogens, plant viruses, and the like.

Lipoxygenase

A nucleic acid sequence encoding a LOX polypeptide from sunflower is also provided. The sunflower LOX polypeptide shares homology with other known LOX proteins from potato, tomato, cowpea, *Arabidopsis*, and rice.

The LOX protein has been implicated in a number of important plant developmental processes. LOX catalyzes the hydroperoxidation of polyunsaturated fatty acids containing *cis, cis*-1,4-pentadiene-conjugated double-bonds. The primary products of LOX-catalyzed reactions are fatty acid hydroperoxides, which are typically metabolized into molecules with known or suspected regulatory activity. For example, LOX derived fatty acid hydroperoxides are precursors to traumatin and jasmonic acid. Traumatin induces cell division and may be involved in the plant wounding response (Zimmerman *et al.* (1979) *Plant Physiol. 63*:536-541). Jasmonates have been implicated as signal transduction molecules in the response of plants to stress, particularly wounding and pathogen attack (Farmer *et al.* (1992) *Cell 4*:129-134). Therefore, the sunflower LOX gene may play an important role in cell division and defense signal transduction pathways that are regulated by the biosynthesis of traumatin and jasmonic acid.

The LOX gene has also been implicated in the regulation of coordinated gene activation in response to wounding. It is speculated that resistance to pathogen attack is the result of the coordinated accumulation of secondary metabolites and protein products. Some of these products, such as proteinase inhibitors, may directly interfere with digestibility of the injected tissue whereas others products may affect food intake. A potato LOX gene, 13-LOX, has been shown to control the expression levels of proteinase inhibitors in a wounding response to insect feeding (Royo *et al.* (1999) *Proc. Natl. Acad. Sci. USA 96:*1146-1151). Therefore, the LOX sequences of the present invention may find use in an antifeedant stragedy by regulating proteinase inhibitor levels in plants, and thereby controlling insect and nematode pathogens.

Additionally, LOX-derived fatty acid hydroperoxides and free radical species are cytotoxic and are capable of damaging membranes, proteins, and DNA (Hildebrand *et al.* (1998) *Curr. Top. Plant Biochem. Physiol. 7*:201-219). Therefore, LOXs may play a role in membrane degradation observed during senescence, wounding, and the hypersensitive response to pathogen attack.

LOX proteins may also play an important role in plant growth and development. There is a positive correlation between LOX activity levels within an organ and its rate of elongation. The concomitant increase in LOXs and the enzymes involved in the metabolism of LOX-derived fatty hydroperoxides is consistent with a role for LOX in generating lipid-derived growth regulators.

Furthermore, in plants, the LOX proteins may be involved in lipid turnover and fat mobilization. Hence, the compositions and methods of the invention find use the turnover of lipids in the developing seedling.

Hence, the LOX sequences of the present invention may be used to modulate many important developmental processes, such as, cell division, seed germination, plant growth and senescence, and/or to enhanced plant resistance to environmental stresses, such as, wounding and pathogen attacks.

The present invention also provides the nucleotide sequences of the LOX promoter. The promoter sequence contains *cis*-elements that resemble W-boxes, TTGACC (nucleotides 322-327 of SEQ ID NO: 5), and G-boxes, CACGTG (nucleotides 722-727 of SEQ ID NO: 5).

G-box and G-box like sequences are involved in the regulation of a variety of unrelated genes and activate transcription in response to various stimuli including: exposure to visible and UV light (Chattopadhyay *et al.* (1998) *Plant Cell 10*:673-683), dehydration-stress (Lam *et al.* (1991) *J Biol Chem 266*: 17131-17135 and Dolferus *et al.* (1994) *Plant Physiol 105*: 1075-1087), cold stress (Dolferus *et al.* (1994) *Plant Physiol 105*: 1075-1087), abscisic acid (Marcotte *et al.* (1989) *Plant Cell 1*: 969-976), sucrose (Urwin *et al.* (1997) *Plant Mol Biol 35*:929-942), and plant pathogen defense response (Wolfgang *et al.* (1997) *EMBO Journal 16*:726-738). In addition, G-box-like sequences were also found to determine tissue-preferred expression patterns (Salinas *et al.* (1992) *Plant Cell 4:* 1485-1493 and Thomas (1993) *Plant Cell 5:* 1401-1410). Functional

analysis of G-box containing promoters has shown that the nucleotide sequences immediately flanking the G-box and/or additional *cis*-acting promoter elements are often required for the G-box to influence transcription activation.

The W-box promoter elements are involved in elicitor-induced gene expression. W-boxes have been identified in a several plant promoters including, for example, members of the WRKY family (Eulgem *et al.* (1999) *EMBO J. 18*:4689-4699) and members of the pathogenesis-related protein family (Rushton *et al.* (1996) *EMBO J. 15*:5690-5700). The fungal elicitor responsiveness of these genes is mediated mainly by the presence of the W-boxes in the promoter elements.

Hence, the LOX promoter sequences find use in the regulated expression of an operably linked heterologous gene of interest. More specifically, the nucleotide sequence may find use as an inducible promoter, more specifically, a pathogen-inducible promoter.

Alcohol Dehydrogenase

A nucleic acid sequence encoding an ADH protein from sunflower is also provided. Sequences of the sunflower alcohol dehydrogenase protein (ADH) share about 85-95% sequence homology with plant alcohol dehydrogenases from garden lettuce, potato, tomato, apple, and maize. ADH is an important enzyme in anaerobic metabolism, and it is usually encoded by a small multigene family in flowering plants. In both maize and *Arabidopsis,* the gene is expressed in seeds, roots, and pollen grains, whereas green aerial plant parts are devoid of detectable ADH activity.

ADH has been implicated in responses to a number of environmental stresses, including low oxygen, drought, salinity, cold acclimation, freezing tolerance, flooding, and wounding. See, for example, Zeevaart *et al.* (1988) *Annu. Rev. Plant Physiol. Plant Mol. Biol. 39*:439-473; Sanchez *et al.* (1991) *Abscisic Acid, Physiology and Biochemistry,* Bio Scientific Publishers, Oxford, UK, pp. 210-216; and Bruxelles *et al.* (1996) *Plant Physiol. 111*:381-391. The ADH sequence of the present invention may find use in modulating a plant's response to adverse environmental stresses.

The sunflower ADH sequences may also find use in modulating the developmental process of fruit ripening. ADH reduces aldehydes to alcohols. Modulation of ADH levels in ripening fruit has been shown to influence the balance

between some of the aldehydes and the corresponding alcohols associated with flavor production. Hence, the compositions and methods of the present invention may find use in the modulation of ADH protein levels leading to a more intense "ripe fruit" flavor. See, for example, Speirs *et al.* (1998) *Plant Physiol. 117*:1047-1058.

The ADH sequences of the invention may additionally find use in enhancing plants defense response. Under low oxygen conditions, i.e., a hypotic state, ADH plays a crucial role in cell survival. ADH serves as the major terminal dehydrogenase in regenerating oxidizing power in mature roots, thereby allowing glycolysis to continue in the absence of oxygen. Treatment of a plant with an elicitor increases the levels of active oxygen species in the plant cells and leads to a transient state of oxidative stress. See, for example, Robertson *et al.* (1995) *Plant Molecular Biology 27*:59-67. Since aerobic respiration is compromised as a result of elicitor action, the ADH sequences of the present invention may find use in modulating a plant's defense against pathogens.

Sclerotinia-inducible protein-1 (SCIP-1)

The nucleic acid sequence encoding a novel sunflower protein, designated *Sclerotinia*-Inducible Protein-1, SCIP-1, is also provided. SCIP-1 has limited homology with hypothetical proteins from several bacteria.

Transcript levels of SCIP-1 increase in both lesion mimic transgenic plants and *Sclerotinia*-infected plants. The accumulation of SCIP-1 in lesion mimic and infected sunflower plants implicates that the protein is involved in the plant defense response to *Sclerotinia* and other pathogens. Hence, the compositions and methods of the invention may find uses for enhancing resistance to plant pathogens, including fungal pathogens, plant viruses, and the like.

Furthermore, a PSI-Blast search revealed that the SCIP-1 sequence of the invention fall into a class of flowering-related plant proteins (CEN and others), as well as some phosphatidylethanolamine-binding proteins (PEBP). The CEN-related proteins are known to be related to a class of phosphatidylethanolamine-binding proteins (PEBP). Banfield *et al.* ((2000) *J Mol Biol 297*:1159-70) determined the crystal structure of the centroradialis protein from *Anthirrhinum*. The structure confirmed what had been suspected by sequence homology studies: that the CEN plant proteins are a subset of the

family of PEBPs. Mammalian forms of PEBP are involved in inhibition of MAP kinase signaling, which is a central signaling cascade regulating cell differentiation (Banfield *et al.* (2000) *J Mol Biol 297*:1159-70). The structure of these proteins suggests that they may play a role in membrane signal transduction (Banfield *et al.* (1998) *Structure 6:*1245-54). In addition, another recent study (Kuramitsu *et al.* (2000) *Electrophoresis 21*:660-4) showed that a line of mammalian cells resistant to tumor necrosis factor-alpha contained elevated levels of a protein identified as a PEBP. The report suggested that this PEBP could be responsible for the resistance of certain cell lines to tumor necrosis factor induced cell death. Hence, the SCIP-1 polypeptide of the invention may play a role in signaling, membrane transduction, or in the regulation of cell death.

Furthermore, flowering plants exhibit two types of inflorescence architecture: determinate and indeterminate. The centroradialis mutation causes the normally indeterminate inflorescence of *Antirrhinum* to terminate in a flower. CEN-related protein have therefore been shown to influence maintenance of the indeterminate state of inflorescence meristems (Pnueli *et al.* (1998) *Development 125*:1979-1989; Bradley *et al.* (1996) *Nature 379*:791-7; Bradley-Desmond *et al.* (1997) *Science 275*:80-83; and Amaya *et al.* (1999) *Plant Cell 11:*1405-1417). In addition, the SCIP-1 shares homology to Terminal Flower 1 (TFL1) from both *Arabidopsis thaliana* and *Brassica.* TFL1 has also been shown to influence inflorescence meristem identity. See, for example, Mimida-Naozumi *et al.* (1999) *Plant-Science 142:* 155-162 and Ohshima *et al.* (1997) *Mol Gen Genet 254:*186-94. Hence, the SCIP-1 sequences of the invention find use in influencing the state of inflorescence of meristem development.

The present invention also provides the nucleotide sequences of the SCIP-1 promoter. The promoter sequence contains *cis*-elements that resemble W-boxes, GTCAA (nucleotides 364-368 and 371-375 of SEQ ID NOS: 8 and 10), and G-boxes, CACGTG (nucleotides 415-420 of SEQ ID NOS: 8 and 10). As with the LOX promoter sequences, the SCIP-1 promoter sequences may find use in the regulated expression of an operably linked heterologous gene of interest. More specifically, the nucleotide sequence may find use as an inducible promoter, more specifically, a pathogen-inducible promoter.

Compositions

Compositions of the invention include the polynucleotide sequences of the sunflower rhoGAP, LOX, ADH, and SCIP-1 genes. In addition, the LOX and SCIP-1 promoter nucleotide sequences are also provided. The polypeptides encoded by those sequences may be involved in various plant developmental processes, including the plant pathogen defense response.

In particular, the present invention provides for isolated nucleic acid molecules comprising nucleotide sequences encoding the amino acid sequences shown in SEQ ID NOS: 2, 4, 7, and 9 or the nucleotide sequences encoding the DNA sequences deposited in a bacterial host as Patent Deposit Nos. PTA-284 and PTA-285 (DNA sequences corresponding to rhoGAP), PTA-286 (DNA sequences corresponding to ADH), PTA-287 (DNA sequences corresponding to LOX), PTA-288 (DNA sequences corresponding to SCIP-1), or the DNA sequences obtained from the overlapping clones deposited in a bacterial host as Patent Deposit Nos. PTA-284 and PTA-285. Further provided are polypeptides having an amino acid sequence encoded by a nucleic acid molecule described herein, for example those set forth in SEQ ID NOS: 1, 3, 6, and 8 or those deposited in a bacterial host as Patent Deposit Nos. PTA-284, PTA-285, PTA-286, PTA-287, PTA-288 and fragments and variants thereof.

The present invention further provides for isolated nucleic acid molecules comprising nucleotide sequences shown in SEQ ID NO: 5 and SEQ ID NO: 10, or nucleotide sequences encoding the DNA sequences deposited in a bacterial host as Patent Deposit Nos. PTA-559 and PTA-1721, and fragments and variants thereof.

By "DNA sequences obtained from the overlapping clones" is intended that the complete DNA sequence of the rhoGAP sequence of the invention (SEQ ID NO: 1) can be obtained by sequencing the two individual clones that together comprise the entire rhoGAP sequence.

Plasmids containing the nucleotide sequences of the invention were deposited with the Patent Depository of the American Type Culture Collection (ATCC), Manassas, Virginia, and assigned Accession Nos. PTA-284, PTA-285, PTA-286, PTA-287, PTA-288, PTA-559, and PTA-1721. Plasmids having the Accession Nos. PTA-284, PTA-285, PTA-286, PTA-287, and PTA-288 were deposited on June 30, 1999. The plasmid having

Accession No. PTA-559 was deposited on August 20, 1999, and the plasmid deposited as Accession No. PTA-1721 was deposited on April 26, 2000. Two of these plasmids, designated as Accession No. PTA-284 and Accession No. PTA-285, contained overlapping clones. The plasmids deposited as PTA-284 and PTA-285 comprise the 5' and the 3' end of the rhoGAP sequence, respectively. It is noted, however, that clones deposited as PTA-284 and PTA-285 contain common sequences at the regions where they overlap. One of skill in the art by sequencing the clones and aligning the overlap may obtain the entire sequence of the sunflower rhoGAP. These deposits will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. These deposits were made merely as a convenience for those of skill in the art and are not an admission that a deposit is required under 35 U.S.C. §112.

The invention encompasses isolated or substantially purified nucleic acid or protein compositions. An "isolated" or "purified" nucleic acid molecule or protein, or biologically active portion thereof, is substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized. Preferably, an "isolated" nucleic acid is free of sequences (preferably protein encoding sequences) that naturally flank the nucleic acid (i.e., sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated nucleic acid molecule can contain less than about 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb, or 0.1 kb of nucleotide sequences that naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived. A protein that is substantially free of cellular material includes preparations of protein having less than about 30%, 20%, 10%, 5%, (by dry weight) of contaminating protein. When the protein of the invention or biologically active portion thereof is recombinantly produced, preferably culture medium represents less than about 30%, 20%, 10%, or 5% (by dry weight) of chemical precursors or non-protein-of-interest chemicals.

Fragments and variants of the disclosed nucleotide sequences and proteins encoded thereby are also encompassed by the present invention. By "fragment" is

intended a portion of the nucleotide sequence or a portion of the amino acid sequence and hence protein encoded thereby. Fragments of a nucleotide sequence may encode protein fragments that retain the biological activity of the native protein and hence affect development, developmental pathways, and defense response by retaining rhoGAP-, LOX-, ADH-, or SCIP-1-like activity. Alternatively, fragments of a nucleotide sequence that are useful as hybridization probes generally do not encode fragment proteins retaining biological activity. Thus, fragments of a nucleotide sequence may range from at least about 20 nucleotides, about 50 nucleotides, about 100 nucleotides, and up to the full-length nucleotide sequence encoding the proteins of the invention.

A fragment of a rhoGAP nucleotide sequence that encodes a biologically active portion of a rhoGAP protein of the invention will encode at least 12, 25, 30, 50, 100, 150, or 200 contiguous amino acids, or up to the total number of amino acids present in a full-length rhoGAP protein of the invention (for example, 201 amino acids for SEQ ID NO: 2).

A fragment of a LOX nucleotide sequence that encodes a biologically active portion of a LOX protein of the invention will encode at least 22, 30, 50, 100, 150, 200, 300, 400, 500, 600, 700, 800, 900 contiguous amino acids, or up to the total number of amino acids present in a full-length LOX protein of the invention (for example, 901 amino acids for SEQ ID NO: 3).

A fragment of an ADH nucleotide sequence that encodes a biologically active portion of an ADH protein of the invention will encode at least 92, 100, 150, 200, 250, 300, 350, 400 contiguous amino acids, or up to the total number of amino acids present in a full-length ADH protein of the invention (for example, 381 amino acids for SEQ ID NO: 7).

A fragment of a SCIP-1 nucleotide sequence that encodes a biologically active portion of a SCIP-1 protein of the invention will encode at least 8, 15, 25, 30, 50, 100, or 150 contiguous amino acids, or up to the total number of amino acids present in a full-length SCIP-1 protein of the invention (for example, 168 amino acids for SEQ ID NO: 9).

Fragments of a rhoGAP, LOX, ADH, and SCIP-1 nucleotide sequence that are useful as hybridization probes or PCR primers generally need not encode a biologically active portion of a rhoGAP, LOX, ADH, or SCIP-1 protein. Thus, a fragment of a

rhoGAP, LOX, ADH, or SCIP-1 nucleotide sequence may encode a biologically active portion of a rhoGAP, LOX, ADH, or SCIP-1 protein, or it may be a fragment that can be used as a hybridization probe or PCR primer using methods disclosed below. A biologically active portion of a rhoGAP, LOX, ADH, or SCIP-1 protein can be prepared by isolating a portion of one of the rhoGAP, LOX, ADH, or SCIP-1 nucleotide sequences of the invention, expressing the encoded portion of the rhoGAP, LOX, ADH, or SCIP-1 protein (e.g., by recombinant expression *in vitro*), and assessing the activity of the encoded portion of the rhoGAP, LOX, ADH, or SCIP-1 protein. Nucleic acid molecules that are fragments of a rhoGAP, LOX, ADH, SCIP-1 nucleotide sequence comprise at least 16, 20, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, or 800 nucleotides, or up to the number of nucleotides present in a full-length rhoGAP, LOX, ADH, or SCIP-1 nucleotide sequence disclosed herein (for example, 824 nucleotides for SEQ ID NO: 1, 3806 nucleotides for SEQ ID NO: 3, 1403 nucleotides for SEQ ID NO: 6, and 746 nucleotide sequences for SEQ ID NO: 8).

By "variants" is intended substantially similar sequences. For nucleotide sequences, conservative variants include those sequences that, because of the degeneracy of the genetic code, encode the amino acid sequence of one of the polypeptides of the invention. Naturally occurring allelic variants such as these can be identified with the use of well-known molecular biology techniques, as, for example, with polymerase chain reaction (PCR) and hybridization techniques as outlined below. Variant nucleotide sequences also include synthetically derived nucleotide sequences, such as those generated, for example, by using site-directed mutagenesis but which still encode a rhoGAP, LOX, ADH, or SCIP-1 protein of the invention. Generally, variants of a particular nucleotide sequence of the invention will have at least 40%, 50%, 60%, 70%, generally at least 75%, 80%, 85%, preferably about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, and more preferably about 98%, 99%, or more sequence identity to that particular nucleotide sequence as determined by sequence alignment programs described elsewhere herein using default parameters.

By "variant" protein is intended a protein derived from the native protein by deletion (so-called truncation) or addition of one or more amino acids to the N-terminal and/or C-terminal end of the native protein; deletion or addition of one or more amino

acids at one or more sites in the native protein; or substitution of one or more amino acids at one or more sites in the native protein. Variant proteins encompassed by the present invention are biologically active, that is they continue to possess the desired biological activity of the native protein, hence they will continue to possess rhoGAP, LOX, ADH, or SCIP-1 activity. Such variants may result from, for example, genetic polymorphism or from human manipulation. Biologically active variants of a native rhoGAP, LOX, ADH, or SCIP-1 protein of the invention will have at least 40%, 50%, 60%, 70%, generally at least 75%, 80%, 85%, preferably about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the amino acid sequence for the native protein as determined by sequence alignment programs described elsewhere herein using default parameters. A biologically active variant of a protein of the invention may differ from that protein by as few as 1-15 amino acid residues, as few as 1-10, such as 6-10, as few as 5, as few as 4, 3, 2, or even 1 amino acid residue.

Biological activity of the rhoGAP, LOX, ADH and SCIP-1 polypeptides can be assayed by any method known in the art. Assays to measure the developmental pathways and defense responses that are influenced by the rhoGAP, LOX, ADH and SCIP-1 polypeptides having rhoGAP-, LOX-, ADH-, and SCIP-1-like activity are well known in the art. Furthermore, assays to detect rhoGAP-like activity include GTP binding assays (Borg *et al.* (1994) *Plant Mol. Biol. 27*:175-187); interactions with Rac or Ras (Diekman *et al.* (1995) *EMBO J. 14*:5297-5305 and Van Aelst *et al.* (1996) *EMBO J. 15*:3778-3786); and GTPase and GTPase activating activity assays (Borg *et al.* (1999) *FEBS Letters 453*:341-345). Assays to detect LOX-like activity include, for example, assays to measure LOX enzymatic activity (Maach *et al.* (1997) *Plant Physiol. 114*:1561-1566, Royo *et al.* (1996) *J. Biol. Chem.* 35:21012-21019 and Voros *et al.* (1998) *FEBS Letters 251*:36-44). Assays to detect ADH-like activity include, for example, ADH enzymatic activity assays (Torres *et al.* (1976) *Biochem. Genetics 14*:87-97).

The proteins of the invention may be altered in various ways including amino acid substitutions, deletions, truncations, and insertions. Methods for such manipulations are generally known in the art. For example, amino acid sequence variants of the rhoGAP, LOX, ADH, or SCIP-1 proteins can be prepared by mutations in the DNA. Methods for mutagenesis and nucleotide sequence alterations are well known in the art. See, for

example, Kunkel (1985) *Proc. Natl. Acad. Sci. USA 82*:488-492; Kunkel *et al.* (1987) *Methods in Enzymol. 154*:367-382; US Patent No. 4,873,192; Walker and Gaastra, eds. (1983) *Techniques in Molecular Biology* (MacMillan Publishing Company, New York) and the references cited therein. Guidance as to appropriate amino acid substitutions that do not affect biological activity of the protein of interest may be found in the model of Dayhoff *et al.* (1978) *Atlas of Protein Sequence and Structure* (Natl. Biomed. Res. Found., Washington, D.C.), herein incorporated by reference. Conservative substitutions, such as exchanging one amino acid with another having similar properties, may be preferred.

Thus, the genes and nucleotide sequences of the invention include both the naturally occurring sequences as well as mutant forms. Likewise, the proteins of the invention encompass both naturally occurring proteins as well as variations and modified forms thereof. Such variants will continue to possess the desired rhoGAP-, LOX-, ADH-, or SCIP-1-like activity. Obviously, the mutations that will be made in the DNA encoding the variant must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure. See, EP Patent Application Publication No. 75,444.

The deletions, insertions, and substitutions of the protein sequences encompassed herein are not expected to produce radical changes in the characteristics of the protein. However, when it is difficult to predict the exact effect of the substitution, deletion, or insertion in advance of doing so, one skilled in the art will appreciate that the effect will be evaluated by routine screening assays. That is, the activity can be evaluated by either an enhanced resistance to pathogens or a modulation in a plant developmental process when expression of the protein sequence is altered.

Variant nucleotide sequences and proteins also encompass sequences and proteins derived from a mutagenic and recombinogenic procedure such as DNA shuffling. With such a procedure, one or more different rhoGAP, LOX, ADH, or SCIP-1 coding sequences can be manipulated to create a new rhoGAP, LOX, ADH, or SCIP-1 possessing the desired properties. In this manner, libraries of recombinant polynucleotides are generated from a population of related sequence polynucleotides comprising sequence regions that have substantial sequence identity and can be

homologously recombined *in vitro* or *in vivo*. For example, using this approach, sequence motifs encoding a domain of interest may be shuffled between the rhoGAP, LOX, ADH, or SCIP-1 gene of the invention and other known rhoGAP, LOX, ADH, or SCIP-1 genes to obtain a new gene coding for a protein with an improved property of interest, such as an increased K_m in the case of an enzyme. Strategies for such DNA shuffling are known in the art. See, for example, Stemmer (1994) *Proc. Natl. Acad. Sci. USA 91*:10747-10751; Stemmer (1994) *Nature 370*:389-391; Crameri *et al.* (1997) *Nature Biotech. 15*:436-438; Moore *et al.* (1997) *J. Mol. Biol. 272*:336-347; Zhang *et al.* *(1997) Proc. Natl. Acad. Sci. USA 94*:4504-4509; Crameri *et al.* (1998) *Nature 391*:288-291; and U.S. Patent Nos. 5,605,793 and 5,837,458.

The compositions of the invention also include isolated nucleic acid molecules comprising the promoter nucleotide sequences set forth in SEQ ID NO: 5 and SEQ ID NO: 10. By "promoter" is intended a regulatory region of DNA usually comprising a TATA box (nucleotides 808-901 of SEQ ID NO: 5) capable of directing RNA polymerase II to initiate RNA synthesis at the appropriate transcription initiation site for a particular coding sequence. A promoter may additionally comprise other recognition sequences generally positioned upstream or 5' to the TATA box, referred to as upstream promoter elements, which influence the transcription initiation rate. Such elements include a W-box (nucleotide sequence 322-327 of SEQ ID NO: 5; nucleotide sequence 364-368 and 371-375 of SEQ ID NO: 10) and a G-box (nucleotide sequence 722-727 of SEQ ID NO: 5; nucleotide sequence 415-420 of SEQ ID NO:10). The promoter sequences of the present invention "regulate" (i.e., repress or activate) transcription from the promoter region. The regulation of transcription by the promoter sequences of the present invention is defined herein as "inducible." By "inducible" is intended the ability of the promoter sequences to regulate expression of an operably linked nucleotide sequence in response to a stimulus.

It is recognized that having identified the nucleotide sequences for the promoter regions disclosed herein, it is within the state of the art to isolate and identify additional regulatory element in the 5' untranslated region upstream from the particular promoter regions defined herein. Thus for example, the promoter regions disclosed herein may further comprise upstream regulatory elements that confer tissue-preferred expression of

heterologous nucleotide sequences operably linked to the disclosed promoter sequence. See particularly, Australian Paten No. AU-A-77751/94 and U.S. Patent Nos. 5,466,785 and 5,635,618.

Fragments and variants of the disclosed LOX and SCIP-1 promoter nucleotide sequences are also encompassed by the present invention. By "fragment" is intended a portion of the nucleotide sequence. Fragments of a nucleotide sequence may retain biological activity and hence retain their transcriptional regulatory activity. Thus, for example, less than the entire promoter sequence disclosed herein may be utilized to drive expression of an operably linked nucleotide sequence of interest, such as a nucleotide sequence encoding a heterologous protein. Alternatively, a fragment of promoter sequence may retain the ability to regulate transcription in the presence of a stimulus when operably linked to a heterologous transcriptional initiation region. Alternatively, fragments of a nucleotide sequence that are useful as hybridization probes generally do not retain biological activity. Thus, fragments of a nucleotide sequence may range from at least about 20 nucleotides, about 50 nucleotides, about 100 nucleotides, and up to the full-length nucleotide sequence of the invention.

Thus, a fragment of a LOX or SCIP-1 promoter nucleotide sequence may encode a biologically active portion of the LOX or SCIP-1 promoter, or it may be a fragment that can be used as a hybridization probe or PCR primer using methods disclosed below. A biologically active portion of a LOX or SCIP-1 promoter can be prepared by isolating a portion of one of the LOX or SCIP-1 promoter nucleotide sequences of the invention, and assessing the activity of the portion of the LOX or SCIP-1 promoter. Nucleic acid molecules that are fragments of a LOX or SCIP-1 promoter nucleotide sequence comprise at least 16, 20, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, or 800 nucleotides, or up to the number of nucleotides present in a full-length LOX or SCIP-1 promoter nucleotide sequence disclosed herein (for example, 880 nucleotide for SEQ ID NO: 5; 510 nucleotide for SEQ ID NO: 10). Assays to determine the activity of a promoter sequence are well known in the art. For example, a LOX or SCIP-1 promoter fragment or variant may be operably linked to the nucleotide sequence encoding any reporter protein, such as the β-glucuronidase protein (GUS reporter) or the luciferase protein. The DNA construct is inserted into the genome of a plant or plant cell

and the mRNA or protein level of the reporter sequence is determined. See, for example, Eulgem *et al.* (1999) *EMBO. 18*: 4689-4699.

Thus, isolated sequences that have promoter activity and which hybridize under stringent conditions to the LOX and SCIP-1 sequences disclosed herein, or to fragments thereof, are encompassed by the present invention. Such sequences will be at least about 40% to 50% homologous, about 60%, 65%, or 70% homologous, and even at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more homologous with the disclosed sequences. That is, the sequence identity of sequences may range, sharing at least about 40% to 50%, about 60%, 65%, or 70%, and even at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity.

By "variants" of the promoter sequences is intended substantially similar sequences. For nucleotide sequences naturally occurring variants such as these can be identified with the use of well-known molecular biology techniques, as, for example, with polymerase chain reaction (PCR) and hybridization techniques as outlined below. Variant nucleotide sequences also include synthetically derived nucleotide sequences, such as those generated, for example, by using site-directed mutagenesis. Generally, variants of a particular nucleotide sequence of the invention will have at least about 40%, 50%, 60%, 65%, 70%, generally at least about 75%, 80%, 85%, preferably at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, and more preferably at least about 98%, 99% or more sequence identity to that particular nucleotide sequence as determined by sequence alignment programs described elsewhere herein using default parameters.

The nucleotide sequences of the invention can be used to isolate corresponding sequences from other organisms, particularly other plants, more particularly other monocots. In this manner, methods such as PCR, hybridization, and the like can be used to identify such sequences based on their sequence homology to the sequences set forth herein. Sequences isolated based on their sequence identity to the entire disease resistant sequences set forth herein or to fragments thereof are encompassed by the present invention. Such sequences include sequences that are orthologs of the disclosed sequences. By "orthologs" is intended genes derived from a common ancestral gene and which are found in different species as a result of speciation. Genes found in different

species are considered orthologs when their nucleotide sequences and/or their encoded protein sequences share substantial identity as defined elsewhere herein. Functions of orthologs are often highly conserved among species.

In a PCR approach, oligonucleotide primers can be designed for use in PCR reactions to amplify corresponding DNA sequences from cDNA or genomic DNA extracted from any plant of interest. Methods for designing PCR primers and PCR cloning are generally known in the art and are disclosed in Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York). See also Innis *et al.*, eds. (1990) *PCR Protocols: A Guide to Methods and Applications* (Academic Press, New York); Innis and Gelfand, eds. (1995) *PCR Strategies* (Academic Press, New York); and Innis and Gelfand, eds. (1999) *PCR Methods Manual* (Academic Press, New York). Known methods of PCR include, but are not limited to, methods using paired primers, nested primers, single specific primers, degenerate primers, gene-specific primers, vector-specific primers, partially-mismatched primers, and the like.

In hybridization techniques, all or part of a known nucleotide sequence is used as a probe that selectively hybridizes to other corresponding nucleotide sequences present in a population of cloned genomic DNA fragments or cDNA fragments (*i.e.,* genomic or cDNA libraries) from a chosen organism. The hybridization probes may be genomic DNA fragments, cDNA fragments, RNA fragments, or other oligonucleotides, and may be labeled with a detectable group such as ^{32}P, or any other detectable marker. Thus, for example, probes for hybridization can be made by labeling synthetic oligonucleotides based on the disease resistant sequences of the invention. Methods for preparation of probes for hybridization and for construction of cDNA and genomic libraries are generally known in the art and are disclosed in Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

For example, an entire sequence disclosed herein, or one or more portions thereof, may be used as a probe capable of specifically hybridizing to the corresponding LOX, SCIP-1, rhoGAP, or ADH sequences and messenger RNAs. To achieve specific hybridization under a variety of conditions, such probes include sequences that are

unique among disease resistant sequences and are preferably at least about 10 nucleotides in length, and most preferably at least about 20 nucleotides in length. Such probes may be used to amplify corresponding sequences from a chosen organism by PCR. This technique may be used to isolate additional coding sequences from a desired organism or as a diagnostic assay to determine the presence of coding sequences in an organism. Hybridization techniques include hybridization screening of plated DNA libraries (either plaques or colonies; see, for example, Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

Hybridization of such sequences may be carried out under stringent conditions. By "stringent conditions" or "stringent hybridization conditions" is intended conditions under which a probe will hybridize to its target sequence to a detectably greater degree than to other sequences (e.g., at least 2-fold over background). Stringent conditions are sequence-dependent and will be different in different circumstances. By controlling the stringency of the hybridization and/or washing conditions, target sequences that are 100% complementary to the probe can be identified (homologous probing). Alternatively, stringency conditions can be adjusted to allow some mismatching in sequences so that lower degrees of similarity are detected (heterologous probing). Generally, a probe is less than about 1000 nucleotides in length, preferably less than 500 nucleotides in length.

Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37°C, and a wash in 1X to 2X SSC (20X SSC = 3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55°C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1.0 M NaCl, 1% SDS at 37°C, and a wash in 0.5X to 1X SSC at 55 to 60°C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1X SSC at

60 to 65°C. Duration of hybridization is generally less than about 24 hours, usually about 4 to about 12 hours.

Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the T_m can be approximated from the equation of Meinkoth and Wahl (1984) *Anal. Biochem. 138*:267-284: $T_m = 81.5°C + 16.6 (\log M) + 0.41 (\%GC) - 0.61 (\%\ form) - 500/L$; where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. The T_m is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. T_m is reduced by about 1°C for each 1% of mismatching; thus, T_m, hybridization, and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with $\geq$90% identity are sought, the T_m can be decreased 10°C. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4°C lower than the thermal melting point (T_m); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10°C lower than the thermal melting point (T_m); low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20°C lower than the thermal melting point (T_m). Using the equation, hybridization and wash compositions, and desired T_m, those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a T_m of less than 45°C (aqueous solution) or 32°C (formamide solution), it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) *Laboratory Techniques in Biochemistry and Molecular Biology—Hybridization with Nucleic Acid Probes,* Part I, Chapter 2 (Elsevier, New York); and Ausubel *et al.*, eds. (1995) *Current Protocols in Molecular Biology*, Chapter 2 (Greene Publishing and Wiley-Interscience, New York).

See Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

In general, sequences that have promotor activity or encode a rhoGAP, LOX, ADH or SCIP-1 polypeptide and which hybridize under stringent conditions to the rhoGAP, LOX, ADH or SCIP-1 sequences disclosed herein, or to fragments thereof, are encompassed by the present invention. Such sequences will be at least 40% to 50% homologous, about 60%, 65%, or 70% homologous, and even at least about 75% homologous, and even about 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% homologous or more with the disclosed sequences. That is, the sequence identity of the sequences may range, sharing at least 40% to 50%, about 60%, 65%, or 70%, and even about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity.

The following terms are used to describe the sequence relationships between two or more nucleic acids or polynucleotides: (a) "reference sequence", (b) "comparison window", (c) "sequence identity", (d) "percentage of sequence identity", and (e) "substantial identity".

(a) As used herein, "reference sequence" is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset or the entirety of a specified sequence; for example, as a segment of a full-length cDNA or gene sequence, or the complete cDNA or gene sequence.

(b) As used herein, "comparison window" makes reference to a contiguous and specified segment of a polynucleotide sequence, wherein the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Generally, the comparison window is at least 20 contiguous nucleotides in length, and optionally can be 30, 40, 50, 100, or longer. Those of skill in the art understand that to avoid a high similarity to a reference sequence due to inclusion of gaps in the polynucleotide sequence a gap penalty is typically introduced and is subtracted from the number of matches.

Methods of alignment of sequences for comparison are well known in the art. Thus, the determination of percent identity between any two sequences can be

accomplished using a mathematical algorithm. Non-limiting examples of such mathematical algorithms are the algorithm of Myers and Miller (1988) *CABIOS 4*:11-17; the local homology algorithm of Smith *et al.* (1981) *Adv. Appl. Math. 2*:482; the homology alignment algorithm of Needleman *and Wunsch* (1970) *J. Mol. Biol. 48*:443-453; the search-for-similarity-method of Pearson *and Lipman* (1988) *Proc. Natl. Acad. Sci. 85*:2444-2448; the algorithm of Karlin and Altschul (1990) *Proc. Natl. Acad. Sci. USA 872*264, modified as in Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA 90*:5873-5877.

Computer implementations of these mathematical algorithms can be utilized for comparison of sequences to determine sequence identity. Such implementations include, but are not limited to: CLUSTAL in the PC/Gene program (available from Intelligenetics, Mountain View, California); the ALIGN program (Version 2.0) and GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Version 8 (available from Genetics Computer Group (GCG), 575 Science Drive, Madison, Wisconsin, USA). Alignments using these programs can be performed using the default parameters. The CLUSTAL program is well described by Higgins *et al.* (1988) *Gene 73*:237-244 (1988); Higgins *et al.* (1989) *CABIOS 5*:151-153; Corpet *et al.* (1988) *Nucleic Acids Res. 16*:10881-90; Huang *et al.* (1992) *CABIOS 8*:155-65; and Pearson *et al.* (1994) *Meth. Mol. Biol. 24*:307-331. The ALIGN program is based on the algorithm of Myers and Miller (1988) *supra*. A PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used with the ALIGN program when comparing amino acid sequences. The BLAST programs of Altschul *et al* (1990) *J. Mol. Biol. 215*:403 are based on the algorithm of Karlin and Altschul (1990) *supra*. BLAST nucleotide searches can be performed with the BLASTN program, score = 100, wordlength = 12, to obtain nucleotide sequences homologous to a nucleotide sequence encoding a protein of the invention. BLAST protein searches can be performed with the BLASTX program, score = 50, wordlength = 3, to obtain amino acid sequences homologous to a protein or polypeptide of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST (in BLAST 2.0) can be utilized as described in Altschul *et al.* (1997) *Nucleic Acids Res. 25*:3389. Alternatively, PSI-BLAST (in BLAST 2.0) can be used to perform an iterated search that detects distant relationships between

molecules. See Altschul *et al.* (1997) *supra*. When utilizing BLAST, Gapped BLAST, PSI-BLAST, the default parameters of the respective programs (e.g., BLASTN for nucleotide sequences, BLASTX for proteins) can be used. See http://www.ncbi.hlm.nih.gov. Alignment may also be performed manually by inspection.

Unless otherwise stated, sequence identity/similarity values provided herein refer to the value obtained using GAP Version 10 using the following parameters: % identity using GAP Weight of 50 and Length Weight of 3; % similarity using Gap Weight of 12 and Length Weight of 4, or any equivalent program. By "equivalent program" is intended any sequence comparison program that, for any two sequences in question, generates an alignment having identical nucleotide or amino acid residue matches and an identical percent sequence identity when compared to the corresponding alignment generated by the preferred program.

GAP uses the algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48: 443-453, to find the alignment of two complete sequences that maximizes the number of matches and minimizes the number of gaps. GAP considers all possible alignments and gap positions and creates the alignment with the largest number of matched bases and the fewest gaps. It allows for the provision of a gap creation penalty and a gap extension penalty in units of matched bases. GAP must make a profit of gap creation penalty number of matches for each gap it inserts. If a gap extension penalty greater than zero is chosen, GAP must, in addition, make a profit for each gap inserted of the length of the gap times the gap extension penalty. Default gap creation penalty values and gap extension penalty values in Version 10 of the Wisconsin Genetics Software Package for protein sequences are 8 and 2, respectively. For nucleotide sequences the default gap creation penalty is 50 while the default gap extension penalty is 3. The gap creation and gap extension penalties can be expressed as an integer selected from the group of integers consisting of from 0 to 200. Thus, for example, the gap creation and gap extension penalties can be 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65 or greater.

GAP presents one member of the family of best alignments. There may be many members of this family, but no other member has a better quality. GAP displays four figures of merit for alignments: Quality, Ratio, Identity, and Similarity. The Quality is

the metric maximized in order to align the sequences. Ratio is the quality divided by the number of bases in the shorter segment. Percent Identity is the percent of the symbols that actually match. Percent Similarity is the percent of the symbols that are similar. Symbols that are across from gaps are ignored. A similarity is scored when the scoring matrix value for a pair of symbols is greater than or equal to 0.50, the similarity threshold. The scoring matrix used in Version 10 of the Wisconsin Genetics Software Package is BLOSUM62 (see Henikoff and Henikoff (1989) *Proc. Natl. Acad. Sci. USA* 89:10915).

For purposes of the present invention, comparison of nucleotide or protein sequences for determination of percent sequence identity to the rhoGAP, LOX, ADH, and SCIP-1 sequences disclosed herein is preferably made using the ClustalW program (Version 1.7 or later) with its default parameters or any equivalent program. By "equivalent program" is intended any sequence comparison program that, for any two sequences in question, generates an alignment having identical nucleotide or amino acid residue matches and an identical percent sequence identity when compared to the corresponding alignment generated by the preferred program.

(c) As used herein, "sequence identity" or "identity" in the context of two nucleic acid or polypeptide sequences makes reference to the residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and therefore do not change the functional properties of the molecule. When sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences that differ by such conservative substitutions are said to have "sequence similarity" or "similarity". Means for making this adjustment are well known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a

score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, California).

(d) As used herein, "percentage of sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity.

(e)(i) The term "substantial identity" of polynucleotide sequences means that a polynucleotide comprises a sequence that has at least 70% sequence identity, preferably at least 80%, more preferably at least 90%, and most preferably at least 95%, compared to a reference sequence using one of the alignment programs described using standard parameters. One of skill in the art will recognize that these values can be appropriately adjusted to determine corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning, and the like. Substantial identity of amino acid sequences for these purposes normally means sequence identity of at least 60%, more preferably at least 70%, 80%, 90%, and most preferably at least 95%.

Another indication that nucleotide sequences are substantially identical is if two molecules hybridize to each other under stringent conditions. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. However, stringent conditions encompass temperatures in the range of about 1°C to about 20°C, depending upon the desired degree of stringency as otherwise qualified herein. Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides they encode are substantially identical. This may occur, e.g., when a copy

of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. One indication that two nucleic acid sequences are substantially identical is when the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the polypeptide encoded by the second nucleic acid.

(e)(ii) The term "substantial identity" in the context of a peptide indicates that a peptide comprises a sequence with at least 70% sequence identity to a reference sequence, preferably 80%, more preferably 85%, most preferably at least 90% or 95% sequence identity to the reference sequence over a specified comparison window. Preferably, optimal alignment is conducted using the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol. 48*:443-453. An indication that two peptide sequences are substantially identical is that one peptide is immunologically reactive with antibodies raised against the second peptide. Thus, a peptide is substantially identical to a second peptide, for example, where the two peptides differ only by a conservative substitution. Peptides that are "substantially similar" share sequences as noted above except that residue positions that are not identical may differ by conservative amino acid changes.

Disease and Pests

Compositions and methods for controlling pathogenic agents are provided. The anti-pathogenic compositions comprise sunflower nucleotide and polypeptide sequences. Particularly, the sunflower nucleic acid and amino acid sequences are selected from rhoGAP-1, LOX, ADH, and SCIP-1. Accordingly, the compositions and methods are useful in protecting plants against fungal pathogens, viruses, nematodes, insects and the like.

By "disease resistance" or "pathogen resistance" is intended that the plants avoid the disease symptoms which are the outcome of plant-pathogen interactions. That is, pathogens are prevented from causing plant diseases and the associated disease symptoms, or alternatively, the disease symptoms caused by the pathogen is minimized or lessened. The methods of the invention can be utilized to protect plants from disease, particularly those diseases that are caused by plant pathogens.

By "antipathogenic compositions" is intended that the compositions of the invention have antipathogenic activity and thus are capable of suppressing, controlling, and/or killing the invading pathogenic organism. An antipathogenic composition of the invention will reduce the disease symptoms resulting from pathogen challenge by at least about 5% to about 50%, at least about 10% to about 60%, at least about 30% to about 70%, at least about 40% to about 80%, or at least about 50% to about 90% or greater. Hence, the methods of the invention can be utilized to protect plants from disease, particularly those diseases that are caused by plant pathogens.

Assays that measure antipathogenic activity are commonly known in the art, as are methods to quantitate disease resistance in plants following pathogen infection. See, for example, U.S. Patent No. 5,614,395, herein incorporated by reference. Such techniques include, measuring over time, the average lesion diameter, the pathogen biomass, and the overall percentage of decayed plant tissues. For example, a plant either expressing an antipathogenic polypeptide or having an antipathogenic composition applied to its surface shows a decrease in tissue necrosis (*i.e.,* lesion diameter) or a decrease in plant death following pathogen challenge when compared to a control plant that was not exposed to the antipathogenic composition. Alternatively, antipathogenic activity can be measured by a decrease in pathogen biomass. For example, a plant expressing an antipathogenic polypeptide or exposed to an antipathogenic composition is challenged with a pathogen of interest. Over time, tissue samples from the pathogen-inoculated tissues are obtained and RNA is extracted. The percent of a specific pathogen RNA transcript relative to the level of a plant specific transcript allows the level of pathogen biomass to be determined. See, for example, Thomma *et al.* (1998) *Plant Biology 95*:15107-15111, herein incorporated by reference.

Furthermore, *in vitro* antipathogenic assays include, for example, the addition of varying concentrations of the antipathogenic composition to paper disks and placing the disks on agar containing a suspension of the pathogen of interest. Following incubation, clear inhibition zones develop around the discs that contain an effective concentration of the antipathogenic polypeptide (Liu *et al.* (1994) *Plant Biology 91*:1888-1892, herein incorporated by reference). Additionally, microspectrophotometrical analysis can be used to measure the *in vitro* antipathogenic properties of a composition (Hu *et al.* (1997)

Plant Mol. Biol. 34:949-959 and Cammue *et al.* (1992) *J. Biol. Chem. 267*: 2228-2233, both of which are herein incorporated by reference).

Methods for increasing pathogen resistance in a plant are provided. The methods involve stably transforming a plant with a DNA construct comprising an anti-pathogenic nucleotide sequence of the invention operably linked to promoter that drives expression in a plant. Such methods may find use in agriculture particularly in limiting the impact of plant pathogens on crop plants. The anti-pathogenic nucleotide sequences comprise the sunflower rhoGAP, LOX, ADH, or SCIP-1 nucleic acid molecules. While the choice of promoter will depend on the desired timing and location of expression of the anti-pathogenic nucleotide sequences, preferred promoters include constitutive and pathogen-inducible promoters.

Additionally, the compositions can be used in formulation use for their disease resistance activities. The proteins of the invention can be formulated with an acceptable carrier into a pesticidal composition(s) that is for example, a suspension, a solution, an emulsion, a dusting powder, a dispersible granule, a wettable powder, and an emulsifiable concentrate, an aerosol, an impregnated granule, an adjuvant, a coatable paste, and also encapsulations in, for example, polymer substances.

Additionally, transformed plants, plant cells, plant tissues and seeds thereof are provided.

It is understood in the art that plant DNA viruses and fungal pathogens remodel the control of the host replication and gene expression machinery to accomplish their own replication and effective infection. The present invention may be useful in preventing such corruption of the cell.

As discussed above, the sequences encoding the sunflower rhoGAP, LOX, ADH, and SCIP-1 are involved in many basic biochemical pathways and cellular functions that influence the plant defense response. Hence, the sequences of the invention may find use in disrupting cellular function of plant pathogens or insect pests as well as altering the defense mechanisms of a host plant to enhance resistance to disease or insect pests. While the invention is not bound by any particular mechanism of action, the gene products, probably proteins or polypeptides, function to inhibit or prevent plant diseases in a plant. Such gene products may be anti-pathogenic. It is recognized that the present

invention is not dependent upon a particular mechanism of defense. Rather, the genes and methods of the invention work to increase resistance of the plant to pathogens independent of how that resistance is increased or achieved.

The methods of the invention can be used with other methods available in the art for enhancing disease resistance in plants. Similarly, the plant defense mechanisms described herein may be used alone or in combination with other proteins or agents to protect against plant diseases and pathogens. Although any one of a variety of second nucleotide sequences may be utilized, specific embodiments of the invention encompass those second nucleotide sequences that, when expressed in a plant, help to increase the resistance of a plant to pathogens. It is recognized that such second nucleotide sequences may be used in either the sense or antisense orientation depending on the desired outcome. Other plant defense proteins include those described in WO 99/43823 and WO 99/43821, all of which are herein incorporated by reference.

Additionally, the LOX and SCIP-1 promoter nucleotide sequences disclosed herein are also useful for genetic engineering of plants to express a phenotype of interest. The promoter sequences may be used to regulate expression of any heterologous nucleotide sequence. Alternatively, the LOX or SCIP-1 promoter sequence may be used to drive expression of its native, i.e., naturally occurring, LOX or SCIP-1 gene sequence disclosed herein. In a specific embodiment, the LOX or SCIP-1 promoter sequences are operably linked to an anti-pathogenic nucleotide sequence and drive expression of said sequence in a plant cell. The LOX or SCIP-1 promoter sequences may therefore be used in creating or enhancing pathogen or disease resistance in a transformed plant.

Pathogens of the invention include, but are not limited to, viruses or viroids, bacteria, insects, nematodes, fungi, and the like. Viruses include any plant virus, for example, tobacco or cucumber mosaic virus, ringspot virus, necrosis virus, maize dwarf mosaic virus, etc. Specific fungal and viral pathogens for the major crops include: Soybeans: *Phytophthora megasperma* f.sp. *glycinea*, *Macrophomina phaseolina*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Fusarium oxysporum*, *Diaporthe phaseolorum* var. *sojae (Phomopsis sojae)*, *Diaporthe phaseolorum* var. *caulivora*, *Sclerotium rolfsii*, *Cercospora kikuchii*, *Cercospora sojina*, *Peronospora manshurica*, *Colletotrichum dematium (Colletotichum truncatum)*, *Corynespora cassiicola*, *Septoria*

glycines, *Phyllosticta sojicola*, *Alternaria alternata*, *Pseudomonas syringae* p.v. *glycinea*, *Xanthomonas campestris* p.v. *phaseoli*, *Microsphaera diffusa*, *Fusarium semitectum*, *Phialophora gregata*, Soybean mosaic virus, *Glomerella glycines*, Tobacco Ring spot virus, Tobacco Streak virus, *Phakopsora pachyrhizi*, *Pythium aphanidermatum*, *Pythium ultimum*, *Pythium debaryanum*, Tomato spotted wilt virus, *Heterodera glycines Fusarium solani*; Canola: *Albugo candida*, *Alternaria brassicae*, *Leptosphaeria maculans*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Mycosphaerella brassiccola*, *Pythium ultimum*, *Peronospora parasitica*, *Fusarium roseum*, *Alternaria alternata*; Alfalfa: *Clavibater michiganese subsp. insidiosum*, *Pythium ultimum*, *Pythium irregulare*, *Pythium splendens*, *Pythium debaryanum*, *Pythium aphanidermatum*, *Phytophthora megasperma*, *Peronospora trifoliorum*, *Phoma medicaginis* var. *medicaginis*, *Cercospora medicaginis*, *Pseudopeziza medicaginis*, *Leptotrochila medicaginis*, *Fusarium*, *Xanthomonas campestris* p.v. *alfalfae*, *Aphanomyces euteiches*, *Stemphylium herbarum*, *Stemphylium alfalfae*; Wheat: *Pseudomonas syringae* p.v. *atrofaciens*, *Urocystis agropyri*, *Xanthomonas campestris* p.v. *translucens*, *Pseudomonas syringae* p.v. *syringae*, *Alternaria alternata*, *Cladosporium herbarum*, *Fusarium graminearum*, *Fusarium avenaceum*, *Fusarium culmorum*, *Ustilago tritici*, *Ascochyta tritici*, *Cephalosporium gramineum*, *Collotetrichum graminicola*, *Erysiphe graminis* f.sp. *tritici*, *Puccinia graminis* f.sp. *tritici*, *Puccinia recondita* f.sp. *tritici*, *Puccinia striiformis*, *Pyrenophora tritici-repentis*, *Septoria nodorum*, *Septoria tritici*, *Septoria avenae*, *Pseudocercosporella herpotrichoides*, *Rhizoctonia solani*, *Rhizoctonia cerealis*, *Gaeumannomyces graminis* var. *tritici*, *Pythium aphanidermatum*, *Pythium arrhenomanes*, *Pythium ultimum*, *Bipolaris sorokiniana*, Barley Yellow Dwarf Virus, Brome Mosaic Virus, Soil Borne Wheat Mosaic Virus, Wheat Streak Mosaic Virus, Wheat Spindle Streak Virus, American Wheat Striate Virus, *Claviceps purpurea*, *Tilletia tritici*, *Tilletia laevis*, *Ustilago tritici*, *Tilletia indica*, *Rhizoctonia solani*, *Pythium arrhenomannes*, *Pythium gramicola*, *Pythium aphanidermatum*, High Plains Virus, European wheat striate virus; Sunflower: Orobanche, *Plasmophora halstedii*, *Sclerotinia sclerotiorum*, Aster Yellows, *Septoria helianthi*, *Phomopsis helianthi*, *Alternaria helianthi*, *Alternaria zinniae*, *Botrytis cinerea*, *Phoma macdonaldii*, *Macrophomina phaseolina*, *Erysiphe cichoracearum*, *Rhizopus oryzae*, *Rhizopus arrhizus*, *Rhizopus*

stolonifer, *Puccinia helianthi*, *Verticillium dahliae*, *Erwinia carotovorum* pv. *carotovora*, *Cephalosporium acremonium*, *Phytophthora cryptogea*, *Albugo tragopogonis*; Corn: *Fusarium moniliforme* var. *subglutinans*, *Erwinia stewartii*, *Fusarium moniliforme*, *Gibberella zeae (Fusarium graminearum)*, *Stenocarpella maydi (Diplodia maydis)*, *Pythium irregulare*, *Pythium debaryanum*, *Pythium graminicola*, *Pythium splendens*, *Pythium ultimum*, *Pythium aphanidermatum*, *Aspergillus flavus*, *Bipolaris maydis* O, T *(Cochliobolus heterostrophus)*, *Helminthosporium carbonum* I, II & III *(Cochliobolus carbonum)*, *Exserohilum turcicum* I, II & III, *Helminthosporium pedicellatum*, *Physoderma maydis*, *Phyllosticta maydis*, *Kabatiella-maydis*, *Cercospora sorghi*, *Ustilago maydis*, *Puccinia sorghi*, *Puccinia polysora*, *Macrophomina phaseolina*, *Penicillium oxalicum*, *Nigrospora oryzae*, *Cladosporium herbarum*, *Curvularia lunata*, *Curvularia inaequalis*, *Curvularia pallescens*, *Clavibacter michiganense* subsp. *nebraskense*, *Trichoderma viride*, Maize Dwarf Mosaic Virus A & B, Wheat Streak Mosaic Virus, Maize Chlorotic Dwarf Virus, *Claviceps sorghi*, *Pseudonomas avenae*, *Erwinia chrysanthemi* pv. *zea*, *Erwinia carotovora*, *Corn stunt spiroplasma*, *Diplodia macrospora*, *Sclerophthora macrospora*, *Peronosclerospora sorghi*, *Peronosclerospora philippinensis*, *Peronosclerospora maydis*, *Peronosclerospora sacchari*, *Sphacelotheca reiliana*, *Physopella zeae*, *Cephalosporium maydis*, *Cephalosporium acremonium*, Maize Chlorotic Mottle Virus, High Plains Virus, Maize Mosaic Virus, Maize Rayado Fino Virus, Maize Streak Virus, Maize Stripe Virus, Maize Rough Dwarf Virus; Sorghum: *Exserohilum turcicum*, *Colletotrichum graminicola (Glomerella graminicola)*, *Cercospora sorghi*, *Gloeocercospora sorghi*, *Ascochyta sorghina*, *Pseudomonas syringae* p.v. *syringae*, *Xanthomonas campestris* p.v. *holcicola*, *Pseudomonas andropogonis*, *Puccinia purpurea*, *Macrophomina phaseolina*, *Perconia circinata*, *Fusarium moniliforme*, *Alternaria alternata*, *Bipolaris sorghicola*, *Helminthosporium sorghicola*, *Curvularia lunata*, *Phoma insidiosa*, *Pseudomonas avenae (Pseudomonas alboprecipitans)*, *Ramulispora sorghi*, *Ramulispora sorghicola*, *Phyllachara sacchari*, *Sporisorium reilianum (Sphacelotheca reiliana)*, *Sphacelotheca cruenta*, *Sporisorium sorghi*, Sugarcane mosaic H, Maize Dwarf Mosaic Virus A & B, *Claviceps sorghi*, *Rhizoctonia solani*, *Acremonium strictum*, *Sclerophthona macrospora*, *Peronosclerospora sorghi*, *Peronosclerospora philippinensis*, *Sclerospora graminicola*,

Fusarium graminearum, *Fusarium oxysporum*, *Pythium arrhenomanes*, *Pythium graminicola*, etc.

Nematodes include parasitic nematodes such as root-knot, cyst, lesion, and renniform nematodes, etc.

Insect pests include insects selected from the orders Coleoptera, Diptera, Hymenoptera, Lepidoptera, Mallophaga, Homoptera, Hemiptera, Orthoptera, Thysanoptera, Dermaptera, Isoptera, Anoplura, Siphonaptera, Trichoptera, etc., particularly Coleoptera and Lepidoptera. Insect pests of the invention for the major crops include: Maize: *Ostrinia nubilalis*, European corn borer; *Agrotis ipsilon*, black cutworm; *Helicoverpa zea*, corn earworm; *Spodoptera frugiperda*, fall armyworm; *Diatraea grandiosella*, southwestern corn borer; *Elasmopalpus lignosellus*, lesser cornstalk borer; *Diatraea saccharalis*, surgarcane borer; *Diabrotica virgifera*, western corn rootworm; *Diabrotica longicornis barberi*, northern corn rootworm; *Diabrotica undecimpunctata howardi*, southern corn rootworm; *Melanotus spp.*, wireworms; *Cyclocephala borealis*, northern masked chafer (white grub); *Cyclocephala immaculata*, southern masked chafer (white grub); *Popillia japonica*, Japanese beetle; *Chaetocnema pulicaria*, corn flea beetle; *Sphenophorus maidis*, maize billbug; *Rhopalosiphum maidis*, corn leaf aphid; *Anuraphis maidiradicis*, corn root aphid; *Blissus leucopterus leucopterus*, chinch bug; *Melanoplus femurrubrum*, redlegged grasshopper; *Melanoplus sanguinipes*, migratory grasshopper; *Hylemya platura*, seedcorn maggot; *Agromyza parvicornis*, corn blot leafminer; *Anaphothrips obscrurus*, grass thrips; *Solenopsis milesta*, thief ant; *Tetranychus urticae*, twospotted spider mite; Sorghum: *Chilo partellus*, sorghum borer; *Spodoptera frugiperda*, fall armyworm; *Helicoverpa zea*, corn earworm; *Elasmopalpus lignosellus*, lesser cornstalk borer; *Feltia subterranea*, granulate cutworm; *Phyllophaga crinita*, white grub; *Eleodes, Conoderus*, and *Aeolus spp.*, wireworms; *Oulema melanopus*, cereal leaf beetle; *Chaetocnema pulicaria*, corn flea beetle; *Sphenophorus maidis*, maize billbug; *Rhopalosiphum maidis*; corn leaf aphid; *Sipha flava*, yellow sugarcane aphid; *Blissus leucopterus leucopterus*, chinch bug; *Contarinia sorghicola*, sorghum midge; *Tetranychus cinnabarinus*, carmine spider mite; *Tetranychus urticae*, twospotted spider mite; Wheat: *Pseudaletia unipunctata*, army worm; *Spodoptera frugiperda*, fall armyworm; *Elasmopalpus lignosellus*, lesser cornstalk borer; *Agrotis*

orthogonia, western cutworm; *Elasmopalpus lignosellus*, lesser cornstalk borer; *Oulema melanopus*, cereal leaf beetle; *Hypera punctata*, clover leaf weevil; *Diabrotica undecimpunctata howardi*, southern corn rootworm; Russian wheat aphid; *Schizaphis graminum*, greenbug; *Macrosiphum avenae*, English grain aphid; *Melanoplus femurrubrum*, redlegged grasshopper; *Melanoplus differentialis*, differential grasshopper; *Melanoplus sanguinipes*, migratory grasshopper; *Mayetiola destructor*, Hessian fly; *Sitodiplosis mosellana*, wheat midge; *Meromyza americana*, wheat stem maggot; *Hylemya coarctata*, wheat bulb fly; *Frankliniella fusca*, tobacco thrips; *Cephus cinctus*, wheat stem sawfly; *Aceria tulipae*, wheat curl mite; Sunflower: *Suleima helianthana*, sunflower bud moth; *Homoeosoma electellum*, sunflower moth; *zygogramma exclamationis*, sunflower beetle; *Bothyrus gibbosus*, carrot beetle; *Neolasioptera murtfeldtiana*, sunflower seed midge; Cotton: *Heliothis virescens*, cotton budworm; *Helicoverpa zea*, cotton bollworm; *Spodoptera exigua*, beet armyworm; *Pectinophora gossypiella*, pink bollworm; *Anthonomus grandis grandis*, boll weevil; *Aphis gossypii*, cotton aphid; *Pseudatomoscelis seriatus*, cotton fleahopper; *Trialeurodes abutilonea*, bandedwinged whitefly; *Lygus lineolaris*, tarnished plant bug; *Melanoplus femurrubrum*, redlegged grasshopper; *Melanoplus differentialis*, differential grasshopper; *Thrips tabaci*, onion thrips; *Franklinkiella fusca*, tobacco thrips; *Tetranychus cinnabarinus*, carmine spider mite; *Tetranychus urticae*, twospotted spider mite; Rice: *Diatraea saccharalis*, sugarcane borer; *Spodoptera frugiperda*, fall armyworm; *Helicoverpa zea*, corn earworm; *Colaspis brunnea*, grape colaspis; *Lissorhoptrus oryzophilus*, rice water weevil; *Sitophilus oryzae*, rice weevil; *Nephotettix nigropictus*, rice leafhopper; *Blissus leucopterus leucopterus*, chinch bug; *Acrosternum hilare*, green stink bug; Soybean: *Pseudoplusia includens*, soybean looper; *Anticarsia gemmatalis*, velvetbean caterpillar; *Plathypena scabra*, green cloverworm; *Ostrinia nubilalis*, European corn borer; *Agrotis ipsilon*, black cutworm; *Spodoptera exigua*, beet armyworm; *Heliothis virescens*, cotton budworm; *Helicoverpa zea*, cotton bollworm; *Epilachna varivestis*, Mexican bean beetle; *Myzus persicae*, green peach aphid; *Empoasca fabae*, potato leafhopper; *Acrosternum hilare*, green stink bug; *Melanoplus femurrubrum*, redlegged grasshopper; *Melanoplus differentialis*, differential grasshopper; *Hylemya platura*, seedcorn maggot; *Sericothrips variabilis*, soybean thrips; *Thrips tabaci*, onion thrips; *Tetranychus turkestani*, strawberry

spider mite; *Tetranychus urticae*, twospotted spider mite; Barley: *Ostrinia nubilalis*, European corn borer; *Agrotis ipsilon*, black cutworm; *Schizaphis graminum*, greenbug; *Blissus leucopterus leucopterus*, chinch bug; *Acrosternum hilare*, green stink bug; *Euschistus servus*, brown stink bug; *Delia platura*, seedcorn maggot; *Mayetiola destructor*, Hessian fly; *Petrobia latens*, brown wheat mite; Oil Seed Rape: *Brevicoryne brassicae*, cabbage aphid; *Phyllotreta cruciferae*, Flea beetle; *Mamestra configurata*, Bertha armyworm; *Plutella xylostella*, Diamond-back moth; *Delia* ssp., Root maggots.

Expression of Sequences

The nucleic acid sequences of the present invention can be expressed in a host cell such as bacteria, yeast, insect, mammalian, or preferably plant cells. It is expected that those of skill in the art are knowledgeable in the numerous expression systems available for expression of a nucleic acid encoding a protein of the present invention. No attempt to describe in detail the various methods known for the expression of proteins in prokaryotes or eukaryotes will be made.

As used herein, "heterologous" in reference to a nucleic acid is a nucleic acid that originates from a foreign species, or, if from the same species, is substantially modified from its native form in composition and/or genomic locus by deliberate human intervention. For example, a promoter operably linked to a heterologous structural gene is from a species different from that from which the structural gene was derived, or, if from the same species, one or both are substantially modified from their original form. A heterologous protein may originate from a foreign species, or, if from the same species, is substantially modified from its original form by deliberate human intervention.

By "host cell" is meant a cell, which comprises a heterologous nucleic acid sequence of the invention. Host cells may be prokaryotic cells such as *E. coli*, or eukaryotic cells such as yeast, insect, amphibian, or mammalian cells. Preferably, host cells are monocotyledonous or dicotyledonous plant cells. A particularly preferred monocotyledonous host cell is a maize host cell.

The disease resistant sequences of the invention are provided in expression cassettes or DNA constructs for expression in the plant of interest. The cassette will include 5' and 3' regulatory sequences operably linked to a rhoGAP, LOX, ADH, or

SCIP-1 sequence of the invention. By "operably linked" is intended a functional linkage between a promoter and a second sequence, wherein the promoter sequence initiates and mediates transcription of the DNA sequence corresponding to the second sequence. Generally, operably linked means that the nucleic acid sequences being linked are contiguous and, where necessary to join two protein coding regions, contiguous and in the same reading frame. The cassette may additionally contain at least one additional gene to be cotransformed into the organism. Alternatively, the additional gene(s) can be provided on multiple expression cassettes.

Such an expression cassette is provided with a plurality of restriction sites for insertion of the disease resistant sequence to be under the transcriptional regulation of the regulatory regions. The expression cassette may additionally contain selectable marker genes.

The expression cassette will include in the 5'-3' direction of transcription, a transcriptional and translational initiation region, a disease resistant DNA sequence of the invention, and a transcriptional and translational termination region functional in plants. The transcriptional initiation region, the promoter, may be native or analogous or foreign or heterologous to the plant host. Additionally, the promoter may be the natural sequence or alternatively a synthetic sequence. By "foreign" is intended that the transcriptional initiation region is not found in the native plant into which the transcriptional initiation region is introduced. As used herein, a chimeric gene comprises a coding sequence operably linked to a transcription initiation region that is heterologous to the coding sequence.

While it may be preferable to express the sequences using heterologous promoters, the native promoter sequences may be used. Such constructs would change expression levels of the disease resistant RNA/protein in the plant or plant cell. Thus, the phenotype of the plant or plant cell is altered.

The termination region may be native with the transcriptional initiation region, may be native with the operably linked DNA sequence of interest, or may be derived from another source. Convenient termination regions are available from the Ti-plasmid of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions. See also Guerineau *et al.* (1991) *Mol. Gen. Genet. 262*:141-144; Proudfoot

(1991) *Cell 64*:671-674; Sanfacon *et al.* (1991) *Genes Dev. 5*:141-149; Mogen *et al.* (1990) *Plant Cell 2*:1261-1272; Munroe *et al.* (1990) *Gene 91*:151-158; Ballas *et al.* (1989) *Nucleic Acids Res. 17*:7891-7903; and Joshi *et al.* (1987) *Nucleic Acid Res. 15*:9627-9639.

Where appropriate, the gene(s) may be optimized for increased expression in the transformed plant. That is, the genes can be synthesized using plant-preferred codons for improved expression. Methods are available in the art for synthesizing plant-preferred genes. See, for example, U.S. Patent Nos. 5,380,831, and 5,436,391, and Murray *et al.* (1989) *Nucleic Acids Res. 17*:477-498, herein incorporated by reference.

Additional sequence modifications are known to enhance gene expression in a cellular host. These include elimination of sequences encoding spurious polyadenylation signals, exon-intron splice site signals, transposon-like repeats, and other such well-characterized sequences that may be deleterious to gene expression. The G-C content of the sequence may be adjusted to levels average for a given cellular host, as calculated by reference to known genes expressed in the host cell. When possible, the sequence is modified to avoid predicted hairpin secondary mRNA structures.

The expression cassettes may additionally contain 5' leader sequences in the expression cassette construct. Such leader sequences can act to enhance translation. Translation leaders are known in the art and include: picornavirus leaders, for example, EMCV leader (Encephalomyocarditis 5' noncoding region) (Elroy-Stein *et al.* (1989) *PNAS USA 86*:6126-6130); potyvirus leaders, for example, TEV leader (Tobacco Etch Virus) (Allison *et al.* (1986); MDMV leader (Maize Dwarf Mosaic Virus); *Virology 154*:9-20), and human immunoglobulin heavy-chain binding protein (BiP), (Macejak *et al.* (1991) *Nature 353*:90-94); untranslated leader from the coat protein mRNA of alfalfa mosaic virus (AMV RNA 4) (Jobling *et al.* (1987) *Nature 325*:622-625); tobacco mosaic virus leader (TMV) (Gallie *et al.* (1989) in *Molecular Biology of RNA*, ed. Cech (Liss, New York), pp. 237-256); and maize chlorotic mottle virus leader (MCMV) (Lommel *et al.* (1991) *Virology 81*:382-385). See also, Della-Cioppa *et al.* (1987) *Plant Physiol. 84*:965-968. Other methods known to enhance translation can also be utilized, for example, introns, and the like.

In preparing the expression cassette, the various DNA fragments may be manipulated, so as to provide for the DNA sequences in the proper orientation and, as appropriate, in the proper reading frame. Toward this end, adapters or linkers may be employed to join the DNA fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction sites, or the like. For this purpose, *in vitro* mutagenesis, primer repair, restriction, annealing, resubstitutions, e.g., transitions and transversions, may be involved.

Generally, the expression cassette will comprise a selectable marker gene for the selection of transformed cells. Selectable marker genes are utilized for the selection of transformed cells or tissues. Marker genes include genes encoding antibiotic resistance, such as those encoding neomycin phosphotransferase II (NEO) and hygromycin phosphotransferase (HPT), as well as genes conferring resistance to herbicidal compounds, such as glufosinate ammonium, bromoxynil, imidazolinones, and 2,4-dichlorophenoxyacetate (2,4-D). See generally, Yarranton (1992) *Curr. Opin. Biotech. 3*:506-511; Christopherson *et al.* (1992) *Proc. Natl. Acad. Sci. USA 89*:6314-6318; Yao *et al.* (1992) *Cell 71*:63-72; Reznikoff (1992) *Mol. Microbiol. 6*:2419-2422; Barkley *et al.* (1980) in *The Operon*, pp. 177-220; Hu *et al.* (1987) *Cell 48*:555-566; Brown *et al.* (1987) *Cell 49*:603-612; Figge *et al.* (1988) *Cell 52*:713-722; Deuschle *et al.* (1989) *Proc. Natl. Acad. Aci. USA 86*:5400-5404; Fuerst *et al.* (1989) *Proc. Natl. Acad. Sci. USA 86*:2549-2553; Deuschle *et al.* (1990) *Science 248*:480-483; Gossen (1993) Ph.D. Thesis, University of Heidelberg; Reines *et al.* (1993) *Proc. Natl. Acad. Sci. USA 90*:1917-1921; Labow *et al.* (1990) *Mol. Cell. Biol. 10*:3343-3356; Zambretti *et al.* (1992) *Proc. Natl. Acad. Sci. USA 89*:3952-3956; Baim *et al.* (1991) *Proc. Natl. Acad. Sci. USA 88*:5072-5076; Wyborski *et al.* (1991) *Nucleic Acids Res. 19*:4647-4653; Hillenand-Wissman (1989) *Topics Mol. Struc. Biol. 10*:143-162; Degenkolb *et al.* (1991) *Antimicrob. Agents Chemother. 35*:1591-1595; Kleinschnidt *et al.* (1988) *Biochemistry 27*:1094-1104; Bonin (1993) Ph.D. Thesis, University of Heidelberg; Gossen *et al.* (1992) *Proc. Natl. Acad. Sci. USA 89*:5547-5551; Oliva *et al.* (1992) *Antimicrob. Agents Chemother. 36*:913-919; Hlavka *et al.* (1985) *Handbook of Experimental Pharmacology*, Vol. 78 (Springer-Verlag, Berlin); Gill *et al.* (1988) *Nature 334*:721-724. Such disclosures are herein incorporated by reference.

The above list of selectable marker genes is not meant to be limiting. Any selectable marker gene can be used in the present invention.

A number of promoters can be used in the practice of the invention. The promoters can be selected based on the desired outcome. That is, the nucleic acids can be combined with constitutive, tissue-preferred, or other promoters for expression in plants. Such constitutive promoters include, for example, the core promoter of the Rsyn7 (PCT Application Serial No. US99/03863); Scp1 promoter (U.S. Patent Application Serial No. 09/028,819), rice actin (McElroy *et al.* (1990) *Plant Cell 2*:163-171); ubiquitin (Christensen *et al.* (1989) *Plant Mol. Biol. 12*:619-632 and Christensen *et al.* (1992) *Plant Mol. Biol. 18*:675-689); pEMU (Last *et al.* (1991) *Theor. Appl. Genet. 81*:581-588); MAS (Velten *et al.* (1984) *EMBO J. 3*:2723-2730); ALS promoter (U.S. Application Serial No. 08/409,297), and the like. Other constitutive promoters include, for example, U.S. Patent Nos. 5,608,149; 5,608,144; 5,604,121; 5,569,597; 5,466,785; 5,399,680; 5,268,463; and 5,608,142.

Generally, it will be beneficial to express the gene from an inducible promoter, particularly from a pathogen-inducible promoter. Such promoters include those from pathogenesis-related proteins (PR proteins), which are induced following infection by a pathogen; e.g., PR proteins, SAR proteins, beta-1,3-glucanase, chitinase, etc. See, for example, Redolfi *et al.* (1983) *Neth. J. Plant Pathol. 89*:245-254; Uknes *et al.* (1992) *Plant Cell 4*:645-656; and Van Loon (1985) *Plant Mol. Virol. 4*:111-116. See also the copending application entitled "Inducible Maize Promoters", U.S. Patent Application Serial No. 09/257,583, filed February 25, 1999.

Of interest are promoters that are expressed locally at or near the site of pathogen infection. See, for example, Marineau *et al.* (1987) *Plant Mol. Biol. 9*:335-342; Matton *et al.* (1989) *Molecular Plant-Microbe Interactions 2*:325-331; Somsisch *et al.* (1986) *Proc. Natl. Acad. Sci. USA 83*:2427-2430; Somsisch *et al.* (1988) *Mol. Gen. Genet. 2*:93-98; and Yang (1996) *Proc. Natl. Acad. Sci. USA 93*:14972-14977. See also, Chen *et al.* (1996) *Plant J. 10*:955-966; Zhang *et al.* (1994) *Proc. Natl. Acad. Sci. USA 91*:2507-2511; Warner *et al.* (1993) *Plant J. 3*:191-201; Siebertz *et al.* (1989) *Plant Cell 1*:961-968; U.S. Patent No. 5,750,386 (nematode-inducible); and the references cited therein. Of particular interest is the inducible promoter for the maize PRms gene, whose

expression is induced by the pathogen *Fusarium moniliforme* (see, for example, Cordero *et al.* (1992) *Physiol. Mol. Plant Path. 41*:189-200).

Additionally, as pathogens find entry into plants through wounds or insect damage, a wound-inducible promoter may be used in the constructions of the invention. Such wound-inducible promoters include potato proteinase inhibitor (pin II) gene (Ryan (1990) *Ann. Rev. Phytopath. 28*:425-449; Duan *et al.* (1996) *Nature Biotechnology 14*:494-498); wun1 and wun2, US Patent No. 5,428,148; win1 and win2 (Stanford *et al.* (1989) *Mol. Gen. Genet. 215*:200-208); systemin (McGurl *et al.* (1992) *Science 225*:1570-1573); WIP1 (Rohmeier *et al.* (1993) *Plant Mol. Biol. 22*:783-792; Eckelkamp *et al.* (1993) *FEBS Letters 323*:73-76); MPI gene (Corderok *et al.* (1994) *Plant J. 6(2)*:141-150); and the like, herein incorporated by reference.

Chemical-regulated promoters can be used to modulate the expression of a gene in a plant through the application of an exogenous chemical regulator. Depending upon the objective, the promoter may be a chemical-inducible promoter, where application of the chemical induces gene expression, or a chemical-repressible promoter, where application of the chemical represses gene expression. Chemical-inducible promoters are known in the art and include, but are not limited to, the maize In2-2 promoter, which is activated by benzenesulfonamide herbicide safeners, the maize GST promoter, which is activated by hydrophobic electrophilic compounds that are used as pre-emergent herbicides, and the tobacco PR-1a promoter, which is activated by salicylic acid. Other chemical-regulated promoters of interest include steroid-responsive promoters (see, for example, the glucocorticoid-inducible promoter in Schena *et al.* (1991) *Proc. Natl. Acad. Sci. USA 88*:10421-10425 and McNellis *et al.* (1998) *Plant J. 14(2)*:247-257) and tetracycline-inducible and tetracycline-repressible promoters (see, for example, Gatz *et al.* (1991) *Mol. Gen. Genet. 227*:229-237, and U.S. Patent Nos. 5,814,618 and 5,789,156), herein incorporated by reference.

The method of transformation/transfection is not critical to the instant invention; various methods of transformation or transfection are currently available. Thus, any method, which provides for effective transformation/transfection may be employed. Transformation protocols as well as protocols for introducing nucleotide sequences into plants may vary depending on the type of plant or plant cell, i.e., monocot or dicot,

targeted for transformation. Suitable methods of introducing nucleotide sequences into plant cells and subsequent insertion into the plant genome include microinjection (Crossway *et al.* (1986) *Biotechniques 4*:320-334), electroporation (Riggs *et al.* (1986) *Proc. Natl. Acad. Sci. USA 83*:5602-5606, *Agrobacterium*-mediated transformation (Townsend *et al.*, U.S. Patent No. 5,563,055; Zhao *et al.*, U.S. Patent No. 5,981,840), direct gene transfer (Paszkowski *et al.* (1984) *EMBO J. 3*:2717-2722), and ballistic particle acceleration (see, for example, Sanford *et al.*, U.S. Patent No. 4,945,050; Tomes *et al.* (1995) "Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment," in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed. Gamborg and Phillips (Springer-Verlag, Berlin); and McCabe *et al.* (1988) *Biotechnology 6*:923-926). Also see Weissinger *et al.* (1988) *Ann. Rev. Genet. 22*:421-477; Sanford *et al.* (1987) *Particulate Science and Technology 5*:27-37 (onion); Christou *et al.* (1988) *Plant Physiol. 87*:671-674 (soybean); McCabe *et al.* (1988) *Bio/Technology 6*:923-926 (soybean); Finer and McMullen (1991) *In Vitro Cell Dev. Biol.* 27P:175-182 (soybean); Singh *et al.* (1998) *Theor. Appl. Genet. 96*:319-324 (soybean); Datta *et al.* (1990) *Biotechnology 8*:736-740 (rice); Klein *et al.* (1988) *Proc. Natl. Acad. Sci. USA 85*:4305-4309 (maize); Klein *et al.* (1988) *Biotechnology 6*:559-563 (maize); Tomes, U.S. Patent No. 5,240,855; Buising *et al.*, U.S. Patent Nos. 5,322,783 and 5,324,646; Tomes *et al. (1995)* "Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment," in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed. Gamborg (Springer-Verlag, Berlin) (maize); Klein *et al.* (1988) *Plant Physiol. 91*:440-444 (maize); Fromm *et al.* (1990) *Biotechnology 8*:833-839 (maize); Hooykaas-Van Slogteren *et al.* (1984) *Nature (London) 311*:763-764; Bytebier *et al.* (1987) *Proc. Natl. Acad. Sci. USA 84*:5345-5349 (Liliaceae); De Wet *et al.* (1985) in *The Experimental Manipulation of Ovule Tissues,* ed. Chapman *et al.* (Longman, New York), pp. 197-209 (pollen); Kaeppler *et al.* (1990) *Plant Cell Reports 9*:415-418 and Kaeppler *et al.* (1992) *Theor. Appl. Genet. 84*:560-566 (whisker-mediated transformation); D'Halluin *et al.* (1992) *Plant Cell 4*:1495-1505 (electroporation); Li *et al.* (1993) *Plant Cell Reports 12*:250-255 and Christou and Ford (1995) *Annals of Botany 75*:407-413 (rice); Osjoda *et al.* (1996) *Nature Biotechnology 14*:745-750 (maize via *Agrobacterium tumefaciens*); all of which are herein incorporated by reference.

The cells that have been transformed may be grown into plants in accordance with conventional ways. See, for example, McCormick *et al.* (1986) *Plant Cell Reports 5*:81-84. These plants may then be grown, and either pollinated with the same transformed strain or different strains, and the resulting hybrid having constitutive expression of the desired phenotypic characteristic identified. Two or more generations may be grown to ensure that constitutive expression of the desired phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure constitutive expression of the desired phenotypic characteristic has been achieved.

The present invention may be used for transformation of any plant species, including, but not limited to, monocots and dicots. Examples of plants of interest include, but are not limited to, corn (*Zea mays*), *Brassica* sp. (e.g., *B. napus, B. rapa, B. juncea*), particularly those *Brassica* species useful as sources of seed oil, alfalfa (*Medicago sativa*), rice (*Oryza sativa*), rye (*Secale cereale*), sorghum (*Sorghum bicolor, Sorghum vulgare*), millet (e.g., pearl millet (*Pennisetum glaucum*), proso millet (*Panicum miliaceum*), foxtail millet (*Setaria italica*), finger millet (*Eleusine coracana*)), sunflower (*Helianthus annuus*), safflower (*Carthamus tinctorius*), wheat (*Triticum aestivum*), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), peanuts (*Arachis hypogaea*), cotton (*Gossypium barbadense*, *Gossypium hirsutum*), sweet potato (*Ipomoea batatus*), cassava (*Manihot esculenta*), coffee (*Coffea* spp.), coconut (*Cocos nucifera*), pineapple (*Ananas comosus*), citrus trees (*Citrus* spp.), cocoa (*Theobroma cacao*), tea (*Camellia sinensis*), banana (*Musa* spp.), avocado (*Persea americana*), fig (*Ficus casica*), guava (*Psidium guajava*), mango (*Mangifera indica*), olive (*Olea europaea*), papaya (*Carica papaya*), cashew (*Anacardium occidentale*), macadamia (*Macadamia integrifolia*), almond (*Prunus amygdalus*), sugar beets (*Beta vulgaris*), sugarcane (*Saccharum* spp.), oats, barley, vegetables, ornamentals, and conifers.

Vegetables include tomatoes (*Lycopersicon esculentum*), lettuce (e.g., *Lactuca sativa*), green beans (*Phaseolus vulgaris*), lima beans (*Phaseolus limensis*), peas (*Lathyrus* spp.), and members of the genus *Cucumis* such as cucumber (*C. sativus*), cantaloupe (*C. cantalupensis*), and musk melon (*C. melo*). Ornamentals include azalea (*Rhododendron* spp.), hydrangea (*Macrophylla hydrangea*), hibiscus (*Hibiscus rosasanensis*), roses (*Rosa*

spp.), tulips (*Tulipa* spp.), daffodils (*Narcissus* spp.), petunias (*Petunia hybrida*), carnation (*Dianthus caryophyllus*), poinsettia (*Euphorbia pulcherrima*), and chrysanthemum. Conifers that may be employed in practicing the present invention include, for example, pines such as loblolly pine (*Pinus taeda*), slash pine (*Pinus elliotii*), ponderosa pine (*Pinus ponderosa*), lodgepole pine (*Pinus contorta*), and Monterey pine (*Pinus radiata*); Douglas-fir (*Pseudotsuga menziesii*); Western hemlock (*Tsuga canadensis*); Sitka spruce (*Picea glauca*); redwood (*Sequoia sempervirens*); true firs such as silver fir (*Abies amabilis*) and balsam fir (*Abies balsamea*); and cedars such as Western red cedar (*Thuja plicata*) and Alaska yellow-cedar (*Chamaecyparis nootkatensis*). Preferably, plants of the present invention are crop plants (for example, corn, alfalfa, sunflower, *Brassica*, soybean, cotton, safflower, peanut, sorghum, wheat, millet, tobacco, etc.), more preferably corn and soybean plants, yet more preferably corn plants.

Prokaryotic cells may be used as hosts for expression. Prokaryotes most frequently are represented by various strains of *E. coli;* however, other microbial strains may also be used. Commonly used prokaryotic control sequences which are defined herein to include promoters for transcription initiation, optionally with an operator, along with ribosome binding sequences, include such commonly used promoters as the beta lactamase (penicillinase) and lactose (lac) promoter systems (Chang *et al.* (1977) *Nature 198*:1056), the tryptophan (trp) promoter system (Goeddel *et al.* (1980) *Nucleic Acids Res. 8*:4057) and the lambda derived P L promoter and N-gene ribosome binding site (Shimatake *et al.* (1981) *Nature 292*:128). The inclusion of selection markers in DNA vectors transfected in *E coli.* is also useful. Examples of such markers include genes specifying resistance to ampicillin, tetracycline, or chloramphenicol.

The vector is selected to allow introduction into the appropriate host cell. Bacterial vectors are typically of plasmid or phage origin. Appropriate bacterial cells are infected with phage vector particles or transfected with naked phage vector DNA. If a plasmid vector is used, the bacterial cells are transfected with the plasmid vector DNA. Expression systems for expressing a protein of the present invention are available using *Bacillus sp.* and *Salmonella* (Palva *et al.* (1983) *Gene 22*:229-235); Mosbach *et al.* (1983) *Nature 302*:543-545).

A variety of eukaryotic expression systems such as yeast, insect cell lines, plant and mammalian cells, are known to those of skill in the art. As explained briefly below, a polynucleotide of the present invention can be expressed in these eukaryotic systems. In some embodiments, transformed/transfected plant cells, as discussed *infra,* are employed as expression systems for production of the proteins of the instant invention.

Synthesis of heterologous nucleotide sequences in yeast is well known (Sherman *et al.* (1982) *Methods in Yeast Genetics,* Cold Spring Harbor Laboratory). Two widely utilized yeasts for production of eukaryotic proteins are *Saccharomyces cerevisiae* and *Pichia pastoris.* Vectors, strains, and protocols for expression in *Saccharomyces* and *Pichia* are known in the art and available from commercial suppliers (e.g., Invitrogen). Suitable vectors usually have expression control sequences, such as promoters, including 3-phosphoglycerate kinase or alcohol oxidase, and an origin of replication, termination sequences and the like as desired.

A protein of the present invention, once expressed, can be isolated from yeast by lysing the cells and applying standard protein isolation techniques to the lists. The monitoring of the purification process can be accomplished by using Western blot techniques or radioimmunoassay of other standard immunoassay techniques.

The sequences of the present invention can also be ligated to various expression vectors for use in transfecting cell cultures of, for instance, mammalian, insect, or plant origin. Illustrative cell cultures useful for the production of the peptides are mammalian cells. A number of suitable host cell lines capable of expressing intact proteins have been developed in the art, and include the HEK293, BHK21, and CHO cell lines. Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter (*e.g.* the CMV promoter, a HSV *tk* promoter or *pgk* (phosphoglycerate kinase) promoter), an enhancer (Queen *et al.* (1986) *Immunol. Rev. 89*:49), and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites (*e.g.,* an SV40 large T Ag poly A addition site), and transcriptional terminator sequences. Other animal cells useful for production of proteins of the present invention are available, for instance, from the American Type Culture Collection.

Appropriate vectors for expressing proteins of the present invention in insect cells are usually derived from the SF9 baculovirus. Suitable insect cell lines include mosquito larvae, silkworm, armyworm, moth and *Drosophila* cell lines such as a Schneider cell line (See, Schneider (1987) *J. Embryol. Exp. Morphol. 27*:353-365).

As with yeast, when higher animal or plant host cells are employed, polyadenylation or transcription terminator sequences are typically incorporated into the vector. An example of a terminator sequence is the polyadenylation sequence from the bovine growth hormone gene. Sequences for accurate splicing of the transcript may also be included. An example of a splicing sequence is the VP1 intron from SV40 (Sprague *et al.*(1983) *J. Virol. 45*:773-781). Additionally, gene sequences to control replication in the host cell may be incorporated into the vector such as those found in bovine papilloma virus type-vectors (Saveria-Campo (1985) *DNA Cloning Vol. II a Practical Approach,* D.M. Glover, Ed., IRL Press, Arlington, Virginia, pp. 213-238).

Animal and lower eukaryotic (e.g., yeast) host cells are competent or rendered competent for transfection by various means. There are several well-known methods of introducing DNA into animal cells. These include: calcium phosphate precipitation, fusion of the recipient cells with bacterial protoplasts containing the DNA, treatment of the recipient cells with liposomes containing the DNA, DEAE dextrin, electroporation, biolistics, and micro-injection of the DNA directly into the cells. The transfected cells are cultured by means well known in the art (Kuchler (1997) *Biochemical Methods in Cell Culture and Virology,* Dowden, Hutchinson and Ross, Inc.).

It is recognized that with these nucleotide sequences, antisense constructions, complementary to at least a portion of the messenger RNA (mRNA) for the disease resistant sequences can be constructed. Antisense nucleotides are constructed to hybridize with the corresponding mRNA. Modifications of the antisense sequences may be made as long as the sequences hybridize to and interfere with expression of the corresponding mRNA. In this manner, antisense constructions having 70%, preferably 80%, more preferably 85% sequence identity to the corresponding antisensed sequences may be used. Furthermore, portions of the antisense nucleotides may be used to disrupt the expression of the target gene. Generally, sequences of at least 50 nucleotides, 100 nucleotides, 200 nucleotides, or greater may be used.

The nucleotide sequences of the present invention may also be used in the sense orientation to suppress the expression of endogenous genes in plants. Methods for suppressing gene expression in plants using nucleotide sequences in the sense orientation are known in the art. The methods generally involve transforming plants with a DNA construct comprising a promoter that drives expression in a plant operably linked to at least a portion of a nucleotide sequence that corresponds to the transcript of the endogenous gene. Typically, such a nucleotide sequence has substantial sequence identity to the sequence of the transcript of the endogenous gene, preferably greater than about 65% sequence identity, more preferably greater than about 85% sequence identity, most preferably greater than about 95% sequence identity. See, U.S. Patent Nos. 5,283,184 and 5,034,323; herein incorporated by reference.

The nucleotide sequences of the present invention may also be used in the sense orientation to suppress the expression of endogenous genes in plants. Methods for suppressing gene expression in plants using nucleotide sequences in the sense orientation are known in the art. The methods generally involve transforming plants with a DNA construct comprising a promoter that drives expression in a plant operably linked to at least a portion of a nucleotide sequence that corresponds to the transcript of the endogenous gene. Typically, such a nucleotide sequence has substantial sequence identity to the sequence of the transcript of the endogenous gene, preferably greater than about 65% sequence identity, more preferably greater than about 85% sequence identity, most preferably greater than about 95% sequence identity. See, U.S. Patent Nos. 5,283,184 and 5,034,323; herein incorporated by reference.

In some embodiments, the content and/or composition of polypeptides of the present invention in a plant may be modulated by altering, *in vivo* or *in vitro*, the promoter of a gene to up- or down- regulate gene expression. In some embodiments, the coding regions of native genes of the present invention can be altered via substitution, addition, insertion, or deletion to decrease activity of the encoded enzyme. See, e.g., Kmiec, U.S. Patent 5,565,350; Zarling *et al.*, PCT/US93/03868. And in some embodiments, an isolated nucleic acid (e.g., a vector) comprising a promoter sequence is transfected into a plant cell. Subsequently, a plant cell comprising the promoter operably linked to a polynucleotide of the present invention is selected for by means known to

those of skill in the art such as, but not limited to, Southern blot, DNA sequencing, or PCR analysis using primers specific to the promoter and to the gene and detecting amplicons produced therefrom. A plant or plant part altered or modified by the foregoing embodiments is grown under plant forming conditions for a time sufficient to modulate the concentration and/or composition of polypeptides of the present invention in the plant. Plant forming conditions are well known in the art and discussed briefly, *supra.*

In general, concentration or composition is increased or decreased by at least 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% relative to a native control plant, plant part, or cell lacking the aforementioned recombinant expression cassette. Modulation in the present invention may occur during and/or subsequent to growth of the plant to the desired stage of development. Modulating nucleic acid expression temporally and/or in particular tissues can be controlled by employing the appropriate promoter operably linked to a polynucleotide of the present invention in, for example, sense or antisense orientation as discussed in greater detail, *supra.* Induction of expression of a polynucleotide of the present invention can also be controlled by exogenous administration of an effective amount of inducing compound. Inducible promoters and inducing compounds, which activate expression from these promoters, are well known in the art. In preferred embodiments, the polypeptides of the present invention are modulated in monocots, particularly maize.

Methods of Use for LOX and SCIP-1 Promoter Sequences

The nucleotide sequences for the LOX and SCIP-1 promoters disclosed in the present invention, as well as variants and fragments thereof, are useful in the genetic manipulation of any host cell, preferably plant cell, when assembled with a DNA construct such that the promoter sequence is operably linked to a nucleotide sequence encoding a heterologous protein of interest. In this manner, the nucleotide sequences of the LOX and SCIP-1 promoter of the invention are provided in expression cassettes along with heterologous nucleotide sequences for expression in the host cell of interest.

The promoters for the LOX and SCIP-1 genes may regulate expression of operably linked nucleotide sequences in an inducible manner. That is, expression of the operably linked nucleotide sequences in a host cell (i.e., plant cell) is induced in response

to a stimulus. By "stimulus" is intended a chemical, which may be applied externally or may accumulate in response to another external stimulus; a pathogen, which may, for example, induce expression as a result of invading a plant cell; or other factor such as environmental stresses, including but not limited to, drought, temperature, and salinity.

As such, the stimulus either directly or indirectly regulates the activity (i.e., an increase in initiation or expression) of an inducible promoter. By "direct action" is intended that the stimulus regulates transcription via a direct interaction between the stimulus and the DNA sequence. By "indirect action" is meant that the regulation occurs via an interaction between the stimulus and some other endogenous or exogenous component in the system, the ultimate result of the indirect action being regulation of the inducible promoter. The stimulus can result from a biotic or abiotic stress, including for example, tissue wounding (i.e., insect herbivory, wind, intentional abiotic infliction of tissue injury or wounding for the purpose of experimentation and/or expression analysis); wound-responsive chemicals (i.e., chemicals that result in the activation of wound-response signal transduction pathways, including, various hormones, jasmonic acid, abscissic acid, linolenic acid, ethylene, their chemical analogues, derivatives, precursors, and the like); pathogens (i.e, fungi, bacteria, nematodes, mycoplasmas, viruses, and insects and the like); and various environmental stresses (i.e., heat, drought, cold, reactive oxygen species and/or radiation). Hence, the promoter of the present invention can be used in combination with a nucleotide sequence that enhances disease resistance, and the compositions therefor find use in the defense of a plant against disease, pathogens, and the like.

Synthetic hybrid promoter regions are known in the art. Such regions comprise upstream promoter elements of one nucleotide sequence operably linked to the promoter element of another nucleotide sequence. In an embodiment of the invention, heterologous gene expression is controlled by a synthetic hybrid promoter comprising the LOX or SCIP-1 promoter sequences of the invention, or a variant or fragment thereof, operably linked to upstream promoter element(s) from a heterologous promoter. Upstream promoter elements that are involved in the plant defense system have been identified and may be used to generate a synthetic promoter. See, for example, Rushton *et al.* (1998) *Curr. Opin. Plant Biol. 1*:311-315. Alternatively, a synthetic LOX or SCIP-

1 promoter sequence may comprise duplications of the upstream promoter elements found within the LOX or SCIP-1 promoter sequence. Such elements include, for example the G-box (nucleotides 722-727 of SEQ ID NO: 5; nucleotides 415-420 of SEQ ID NO: 10) or W-box (nucleotides 322-327 of SEQ ID NO: 5; nucleotides 364-368 and 371-375 of SEQ ID NO: 10).

It is recognized that the promoter sequence of the invention may be used with its native LOX or SCIP-1 coding sequences. A DNA construct comprising the LOX or SCIP-1 promoter operably linked with its native LOX or SCIP-1 gene may be used to transform any plant of interest to bring about a desired phenotypic change, such as enhanced disease resistance. Where the promoter and its native gene is naturally occurring within the plant, *i.e.*, in sunflower, transformation of the plant with these operably linked sequences also results in either a change in phenotype, such as enhanced disease resistance, the insertion of operably linked sequences within a different region of the chromosome thereby altering the plant's genome, or the modulation in the level of expression of the nucleotide sequence of interest.

In another embodiment of the invention, expression cassettes will comprise a transcriptional initiation region comprising the LOX or SCIP-1 promoter nucleotide sequences disclosed herein, or variants or fragments thereof, operably linked to the heterologous nucleotide sequence whose expression is to be controlled by the inducible promoter of the invention.

The promoter nucleotide sequences and methods disclosed herein are useful in regulating expression of any heterologous nucleotide sequence in a host plant in order to vary the phenotype of a plant. Various changes in phenotype are of interest including modifying the fatty acid composition in a plant, altering the amino acid content of a plant, altering a plant's pathogen defense mechanism, and the like. These results can be achieved by providing expression of heterologous products or increased expression of endogenous products in plants. Alternatively, the results can be achieved by providing for a reduction of expression of one or more endogenous products, particularly enzymes or cofactors in the plant. These changes result in a change in phenotype of the transformed plant.

Genes of interest are reflective of the commercial markets and interests of those involved in the development of the crop. Crops and markets of interest change, and as developing nations open up world markets, new crops and technologies will emerge also. In addition, as our understanding of agronomic traits and characteristics such as yield and heterosis increase, the choice of genes for transformation will change accordingly. General categories of genes of interest include, for example, those genes involved in information, such as zinc fingers, those involved in communication, such as kinases, and those involved in housekeeping, such as heat shock proteins. More specific categories of transgenes, for example, include genes encoding important traits for agronomics, insect resistance, disease resistance, herbicide resistance, sterility, grain characteristics, and commercial products. Genes of interest include, generally, those involved in oil, starch, carbohydrate, or nutrient metabolism as well as those affecting kernel size, sucrose loading, and the like.

Agronomically important traits such as oil, starch, and protein content can be genetically altered in addition to using traditional breeding methods. Modifications include increasing content of oleic acid, saturated and unsaturated oils, increasing levels of lysine and sulfur, providing essential amino acids, and also modification of starch. Hordothionin protein modifications are described in U.S. Application Serial Nos. 08/838,763, filed April 10, 1997; U.S. Patent Nos. 5,703,049, 5,885,801, and 5,885,802; herein incorporated by reference. Another example is lysine and/or sulfur rich seed protein encoded by the soybean 2S albumin described in U.S. Patent No. 5,850,016, and the chymotrypsin inhibitor from barley, described in Williamson *et al.* (1987) *Eur. J. Biochem. 165*:99-106, the disclosures of which are herein incorporated by reference.

Derivatives of the coding sequences can be made by site-directed mutagenesis to increase the level of preselected amino acids in the encoded polypeptide. For example, the gene encoding the barley high lysine polypeptide (BHL) is derived from barley chymotrypsin inhibitor, U.S. Application Serial No. 08/740,682, filed November 1, 1996, and PCT/US97/20441, filed October 31, 1997, the disclosures of which are herein incorporated by reference. Other proteins include methionine-rich plant proteins such as from sunflower seed (Lilley *et al.* (1989) *Proceedings of the World Congress on Vegetable Protein Utilization in Human Foods and Animal Feedstuffs*, ed. Applewhite

(American Oil Chemists Society, Champaign, Illinois), pp. 497-502; herein incorporated by reference); corn (Pedersen *et al.* (1986) *J. Biol. Chem. 261*:6279; Kirihara *et al.* (1988) *Gene 71*:359; both of which are herein incorporated by reference); and rice (Musumura *et al.* (1989) *Plant Mol. Biol. 12*:123, herein incorporated by reference). Other agronomically important genes encode latex, Floury 2, growth factors, seed storage factors, and transcription factors.

Insect resistance genes may encode resistance to pests that have great yield drag such as rootworm, cutworm, European Corn Borer, and the like. Such genes include, for example, *Bacillus thuringiensis* toxic protein genes (U.S. Patent Nos. 5,366,892; 5,747,450; 5,736,514; 5,723,756; 5,593,881; and Geiser *et al.* (1986) *Gene 48*:109); lectins (Van Damme *et al.* (1994) *Plant Mol. Biol. 24*:825); and the like.

Genes encoding disease resistance traits include detoxification genes, such as against fumonosin (U.S. Patent No. 5,792,931); avirulence (avr) and disease resistance (R) genes (Jones *et al.* (1994) *Science 266*:789; Martin *et al.* (1993) *Science 262*:1432; and Mindrinos *et al.* (1994) *Cell 78*:1089); and the like.

Herbicide resistance traits may include genes coding for resistance to herbicides that act to inhibit the action of acetolactate synthase (ALS), in particular the sulfonylurea-type herbicides (e.g., the acetolactate synthase (ALS) gene containing mutations leading to such resistance, in particular the S4 and/or Hra mutations), genes coding for resistance to herbicides that act to inhibit action of glutamine synthase, such as phosphinothricin or basta (e.g., the bar gene), or other such genes known in the art. The *bar* gene encodes resistance to the herbicide basta, the *nptII* gene encodes resistance to the antibiotics kanamycin and geneticin, and the ALS-gene mutants encode resistance to the herbicide chlorsulfuron.

Sterility genes can also be encoded in an expression cassette and provide an alternative to physical detasseling. Examples of genes used in such ways include male tissue-preferred genes and genes with male sterility phenotypes such as QM, described in U.S. Patent No. 5,583,210. Other genes include kinases and those encoding compounds toxic to either male or female gametophytic development.

The quality of grain is reflected in traits such as levels and types of oils, saturated and unsaturated, quality and quantity of essential amino acids, and levels of cellulose. In

corn, modified hordothionin proteins, described in U.S. Application Serial No. 08/838,763 (filed April 10, 1997) and U.S. Patent Nos. 5,703,049, 5,885,801, and 5,885,802, provide descriptions of modifications of proteins for desired purposes.

Commercial traits can also be encoded on a gene or genes that could increase for example, starch for ethanol production, or provide expression of proteins. Another important commercial use of transformed plants is the production of polymers and bioplastics such as described in U.S. Patent No. 5,602,321. Genes such as β-Ketothiolase, PHBase (polyhydroxyburyrate synthase), and acetoacetyl-CoA reductase (see Schubert *et al.* (1988) *J. Bacteriol. 170*:5837-5847) facilitate expression of polyhyroxyalkanoates (PHAs). Exogenous products include plant enzymes and products as well as those from other sources including procaryotes and other eukaryotes. Such products include enzymes, cofactors, hormones, and the like. The level of proteins, particularly modified proteins having improved amino acid distribution to improve the nutrient value of the plant, can be increased. This is achieved by the expression of such proteins having enhanced amino acid content.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

Example 1. The Isolation of Sunflower Disease Resistant Genes and the LOX and SCIP-1 Promoter Sequences.

Materials and Methods

Plant Material

Sunflower plants were grown in the greenhouse and growth chamber. The sunflower line SMF 3 and oxox-transgenic sunflower were used for an RNA profiling study by CuraGen. Sunflower pathogen, *Sclerotinia sclerotiorum* was maintained on PDA plate at 20 °C in dark.

Preparation of Total RNA for RNA Profiling Study and Northern Analysis

Plant materials were ground in liquid nitrogen and total RNA was extracted by the Tri-agent Method. For each RNA profiling study, RNA samples from 6-week-old sunflower leaves and stems of transgenic sunflower plants expressing a wheat oxalate oxidase gene (oxox) were compared with RNA samples from the non-transformed parent sunflower line SMF3. Total RNA (20 ug) was separated in a 1% agarose gel containing formaldehyde. Ethidium bromide was included to verify equal loading of RNA. After transfer onto Hybond N+ membrane, the blots were hybridized with ^{32}P-labelled rhoGAP, LOX, ADH, or SCIP-1 cDNA probes. A duplicate blot was hybridized with a ribosomal 18S RNA probe as a control. Hybridization and washing conditions were performed according to Church and Gilbert.

RNA Profiling

Differences in the expression of specific genes between sunflower plants expressing a wheat oxalate oxidase gene and the sunflower line SMF3 were determined using gene expression profiling. Total RNA was analyzed using the gene expression profiling process (GeneCalling®) as described in U.S. Patent No. 5,871,697, herein incorporated by reference.

Isolation of Full-Length or Flanking Sequences by PCR Amplification of cDNA Ends

The four cDNAs of the present invention were isolated by using RNA profiling and PCR-based technologies. RNA profiling studies were conducted through the collaboration with CuraGen Corp. The sequence information generated by the CuraGen study was used to design gene specific primers to amplifying both 3' and/or 5' end regions of the target genes using PCR-based RACE technology. *Sclerotinia*-infected and oxox-induced cDNA libraries or cDNAs made by the Marathon cDNA Amplification Kit (Clontech) were used as template for PCR amplification. To facilitate cloning full-length cDNAs from initial cloned regions, a pair of 28 bp vector primers were designed that flanked the cDNAs on both ends (3' and 5') of the pBS vector. Amplification of either the 5' or 3' end of the cDNA was accomplished using a vector primer (pBS-upper or pBS-lower) paired with a gene specific primer. The 5' end of a specific gene with the intact

ATG start codon was cloned and sequenced. The full-length cDNA was amplified by using a new gene specific 5' primer containing sequences upstream of the ATG start codon and a 3' primer containing vector sequences.

PCR reactions were performed in a total volume of 25 µl in 10 mM Tris-HCL, pH 8.3; 1.5 mM $MgCl_2$; 50 mM KCl; 0.1 mM dNTPs; 0.25 µM of each primer with 0.5 units of advantage cDNA polymerase mix (Clontech) or Pwo DNA polymerase (Boehringer). Genomic DNA and/or cDNA library mixtures were used as template for PCR amplification.

Isolation of Disease Inducible Promoters

The promoter regions of the LOX and SCIP-1 genes were isolated from sunflower genomic DNA using Universal Genomic Walker Kit (clontech) according to the manufactures instructions. Restriction digested genomic DNAs were ligated with an adapter to construct pools of genomic DNA fragments for walking by PCR.

Analysis of Amplified PCR Products

Amplified PCR fragments with the expected sizes were individually sliced out of the gel for second round PCR re-amplification. The second round of PCR was carried out using the same conditions as initial PCR. Each second round PCR product that showed a single band of the expected size was cloned into the TA vector (Clontech) according to the manufacturer's instructions. Positive clones were selected for DNA sequencing using an Applied Biosystems 373A (ABI) automated sequencer at the Nucleic Acid Analysis Facility of Pioneer Hi-Bred Int'l Inc. DNA sequence analysis was carried out with the Sequencer (3.0). Multiple-sequence alignments (Clustal W) of the DNA sequence were carried out with the Curatool (CuraGen).

Construction of the Sclerotinia-Infected and Resistance-Enhanced (Oxox-Induced) Sunflower cDNA Libraries

Six-week old SMF3 sunflower plants were infected with *Sclerotinia sclerotrium* by petiole inoculation with *Sclerotinia*-infected carrot plugs. Six days after infection, leaf and stem tissues were collected from infected plants for total RNA isolation. Total RNA

was also isolated from 6-week-old sunflower oxox-transgenic plants (line 610255) expressing a wheat oxalate oxidase gene. Our previous studies have shown that elevated levels of H_2O_2, SA, and PR1 protein were detected in oxox-transgenic plants at the 6-week stage and that the plants showed more resistance to *Sclerotinia* infection. The mRNAs were isolated using a mRNA purification kit (BRL) according to the manufacture's instructions. The cDNA libraries were constructed using the ZAP-cDNA synthesis kit and the pBluescript phagemid (Stratagene). A cDNA library mixture for PCR cloning was made of oxox-transgenic stem and *Sclerotinia*-infected leaf libraries (1:2 mix).

Fungal Infection and Chemical Treatments

Sunflower plants SMF3 were planted in 4-inch pots and grown in greenhouse for first four weeks. After transfer to growth chamber, plants were maintained under 12-hour photoperoid at 22°C with an 80% relative humidity. Six-week old plants were inoculated with *Sclerotinia*-infected carrot plugs or sprayed with four different chemicals at the given concentration. For each plant, three petioles were inoculated and wrapped with 1x2 inch parafilm. Plant tissue samples were harvested at different time points and immediately frozen in liquid nitrogen and then stored at –80°C.

CuraGen Analysis and Database Search

An RNA profiling study identified 7 bands that were induced in oxox-transgenic and *Sclerotinia*-infected sunflower plants (Table 1).

Table 1. Summary of RNA profiling results from four sets of experiments.

Gene name	Fold Diff	Band Id	Stem&Leaf Oxox-48d	Leaf Oxox-48d	Leaf Infection	Stem Infection
GTPase-activating protein (rhoGAP)	+2.6	m0v0-120.8	**+2.58**	+3.15	0	0
Lipoxygenase	+11.8	m1n0-257.6	0	0	0	**+11.82**
Lipoxygenase	+6.4	w0h0-279.1	0	0	0	**+6.42**
Lipoxygenase	+3.4	m0r0-276.7	0	0	0	**+3.37**
Alcohol dehydrogenase	+7.7	i0a0-289.5	**+7.69**	0	0	+2.21
SCIP-1	-+3.2	l0m0-273.9	0	0	**+23.24**	0
SCIP-1	+12.3	l0m0-94.2	0	+.74	**+12.28**	0

Note: three lipoxygenase bands belong to same isolated sunflower LOX cDNA and the two SCIP-1 bands are the same cDNA.

The CuraGen band m0v0-120.8 was induced 2 to 3 fold in oxox-transgenic plants and showed lesion mimic phenotype (Table 1). The translated product of CuraGen band m0v0-120.8 shares (amino acids 23-118 of SEQ ID NO: 2) 78% amino acid identity with an *Arabidopsis* hypothetical 23.6 kDa protein (Accession No. O81806). Band m0v0-120.8 represents amino acids 23-118 of SEQ ID NO: 2. The nucleotide sequence encoding the rhoGAP polypeptide is shown in SEQ ID NO: 1. The sunflower rhoGAP clone is 824 bp long with an ORF from nucleotides 35 to nucleotide 637 from the 5' end. It encodes a 201 amino acid protein (SEQ ID NO: 2) with a molecular weight of 23.4 kDa and pI of 8.1. A BLASTX (version 2.0) search indicated that the sunflower rhoGAP-1 protein has sequence homology (about 40%) with human p50rhoGAP (Accession No. Z23024) and also shares sequence homology with the *Lotus japonicus* rhoGAP (Accession No. AF064787).

Three of the CuraGen bands (m1n0-257.6, w0h0-279.1 and m0r0-276.7) were identified as lipoxygenase homologues by blast search of Genbank. The bands were induced 3.37- to 11.82- fold higher in *Sclerotinia*-infected samples compared with uninfected control samples. The sunflower LOX nucleotide sequence is shown in SEQ ID NO: 3. The LOX ORF extends from nucleotide 881 to nucleotide 3583. The LOX ORF encodes a 901 amino acid protein (SEQ ID NO: 4) with a predicted molecular weight of 101.37 kDa and a pI of 5.61. The sunflower LOX protein shares homology to other LOX genes from potato (Accession No. 034370), tomato (Accession No. Q96573), cowpea (Accession No. P93698), *Arabidopsis* (Accession No. LOXC_ARATH) and rice (Accession No. LOXC_ORYSA).

The CuraGen band i0a0-289.5 was induced 7.7-fold higher in *Sclerotinia*-infected samples compared with uninfected control samples. PCR-based cloning of this band allowed the isolation of a cDNA encoding a 381 amino acid polypeptide, which has 85-95% amino acid identity with known plant ADH sequences from garden lettuce (Accession No. Q40249), potato (Accession No. P14674), tomato (Accession No. P28032), apple (Accession No. P48977), and maize (Accession No. P00333).

The CuraGen bands l0m0-273.9 and l0m0-94.2 were induced 23.2- and 12.3- fold higher in *Sclerotinia*-infected samples compared with uninfected control samples,

respectfully. PCR-based cloning of this band allowed two partial cDNA clones to be isolated. The full length cDNA of SCIP-1 encodes a 168 amino acid polypeptide. A BLASTP (version 2.0) sequence alignment showed that SCIP-1 has about 41% identity from amino acid 10-167 with a conserved protein from *Methanobacterium thermoautotrophicum* (Accession No. O26373), about 34% identity from amino acid residues1-163 with a hypothetical 20.7 kDa protein from *Pyrococcus abyssi* (Accession No. CAB50064), about 35% identity from amino acid residues 13-159 with a conserved hypothetical protein from *Archaeoglobus fulgidus* (Accession No. O28575), about 36% identity with a hypothetical 16.5 kDa protein from *Chlamydia trachomatis* (Accession No. O84741), about 36% identity from amino acid residues 21-163, about 36% identity from amino acid 21 to 163 with a long hypothetical protein from *Pyrococcus horikoshii* (Accession No.O58984), about 35% identity with a hypothetical 18.9 kDa protein from *Aquifex aeolicus* (Accession No. O67293), about 35% identity from amino acid residues 21-167 with a hypothetical 17.1 kDa protein from *E. coli* (Accession No. P12994), and about 35% identity from amino acid residues 8-135 with a hypothetical 19.5 kDa protein from *E. coli* (Accession No. P77368).

As discussed further in example 2, the transcript encoding SCIP-1 was up-regulated in infected tissue as compared to control tissue. The accumulation of SCIP-1 in lesion mimic and infected sunflower plants suggests that the polypeptide may be involved in the plant defense response to *Sclerotinia* and other pathogens.

Position specific iterative BLAST (PSI-BLAST) (http://www.ncbi.nlm.nih.gov/Education/BLASTinfo/psi1.html) was performed on the SCIP-1 sequence of the invention. PSI-BLAST refers to a feature of BLAST 2.0 in which a profile (or position specific scoring matrix, PSSM) is constructed (automatically) from a multiple alignment of the highest scoring hits in an initial BLAST search. The PSSM is generated by calculating position-specific scores for each position in the alignment. Highly conserved positions receive high scores and weakly conserved positions receive scores near zero. The profile is used to perform a second (BLAST search and the results of each "iteration" used to refine the profile. The second profile is used to perform another BLAST search and so on, until the profile no longer brings back new information and is said to have “converged”. This iterative searching strategy results

in increased sensitivity. PSI-BLAST searches thus can identify subtle homologies to annotated entries in the database. PSI-BLAST is an important tool for predicting both biochemical activities and function from sequence relationships.

A search was set up using the Lion BioSCOUT implementation of the PSI-Blast algorithm. The query was the SCIP-1 polypeptide sequence, and the NR database provided with Lion BioScout was searched. Parameters were set to match as closely as possible the defaults recommended by NCBI.

The best PSI-Blast hits found in the database for SCIP-1 were the group of archeal and eubacterial hypothetical proteins found in earlier BLASTP searches. As the PSI-Blast search progressed, however, another group of proteins entered the alignments. Table 2 shows the 14 best hits from the PSI-Blast search other than the hypothetical proteins. It is notable that the areas of alignment are found across the entire length of these proteins (data not shown).

The proteins found in the PSI-Blast search fall into a class of flowering-related plant protein (CEN and others), as well as some phosphatidylethanolamine-binding proteins (PEBP). The CEN-related proteins are known to be related to a class of phosphatidylethanolamine-binding proteins (PEBP). The alignment of the SCIP-1 protein with both the CEN proteins and PEBP suggests that SCIP-1 may be related to this class of PEBP proteins. By analogy to other reported PEBP-type proteins, SCIP-1 may play a role in signaling, in membrane transduction, or in the regulation of cell death.

Table 2. Selected hits from PSI-Blast search with SCIP-1 against the NR protein database. From the entire set of PSI-Blast results, the 14 best hits were selected that were not from bacteria, and that had functional annotations associated with the molecule.

5

Hit (Accession Numbers)	PSI-Blast expectation	Description	Reference
trembl|AB017525|AB017525_1	1e-29	gene: "BNTFL1-1"; *Brassica napus* BNTFL1-1 gene, complete cds	Mimida *et al.* (1999) *Plant Science 142*:155-162
swiss|P54186|D1_ONCVO	1e-29	D1 PROTEIN (FRAGMENT) from *Onchocerca volvulus* (nematode)	Erttmann *et al.* (1996) *Gene 174:*203-7
trembl|AB017528|AB017528_1	3e-29	gene: "BRTFL1-1"; *Brassica rapa* BRTFL1-1 gene, complete cds	Mimida *et al.* (1999) *Plant Science 142*:155-162
trembl|AF145261|AF145261_1	1e-28	gene: "CET4"; product: "CEN-like protein 4"; *Nicotiana tabacum* CEN-like protein 4 (CET4) mRNA, complete cds.	Amaya *et al.* (1999) *Plant Cell 11*:1405-1417
trembl|D87130|ATD130_1	2e-28	gene: "terminal flower1"; product: "terminal flower1"; *Arabidopsis thaliana* DNA for terminal flower1, complete cds.	Ohshima *et al.* (1997) *Mol Gen Genet 254:*186-94
trembl|U84140|U84140_1	4e-28	gene: "sp"; product: "self-pruning protein"; *Lycopersicon esculentum* self-pruning protein (sp) mRNA, complete cds	Pnueli *et al.* (1989) *Development 125:*1979-1989
trembl|AB027456|AB027456_1	1e-27	gene: "CiFT"; *Citrus unshiu* CiFT mRNA, complete cds.	Kobayashi *et al.* (1999) *Science 286*:1960-2
swiss|O16264|PBPH_CAEEL	3e-27	PHOSPHATIDYLETHANOLAMINE-BINDING PROTEIN HOMOLOG F40A3.3.	Unpublished
trembl|AB027506|AB027506_1	6e-27	gene: "TSF"; product: "TSF"; *Arabidopsis thaliana* TSF (TWIN SISTER OF FT) mRNA, complete cds	Kobayashi *et al.* (1999) *Science 286*:1960-2
swiss|Q41261|CEN_ANTMA .	3e-26	CENTRORADIALIS PROTEIN. [*Antirrhinum*=snapdragons, mRNA, 929 nt].	Bradley *et al.* (1996) *Nature 379*:791-7 and Bradley *et al.* (1997) *Science 275*:80-83.
trembl|AB027504|AB027504_1	5e-26	gene: "FT"; product: "FT"; *Arabidopsis thaliana* FT (FLOWERING LOCUS T) mRNA, complete cds	Kobayashi *et al.* (1999) *Science 286:*1960-2
trembl|AF159882|AF159882_1	8e-26	gene: "Fdr2"; product: "Cen-like protein FDR2"; *Oryza sativa* Cen-like protein FDR2 (Fdr2) mRNA, complete cds	Unpublished
trembl|AB024712|AB024712_1	1e-24	gene: "ATC"; *Arabidopsis thaliana* ATC (centroradialis) gene, complete cds, strain:Landsberg	Unpublished
trembl|D16111|HSHRPBP_1	1e-23	product: "human homologue of rat phosphatidylethanolamine binding protein"; Human mRNA for human homologue of rat phosphatidylethanolamine binding protein, complete cds	Hori *et al.* (1994) *Gene 140:*293-4

Example 2: Northern Analysis of mRNA Levels of Disease Resistance Genes Following Biotic and Abiotic Stresses

The expression of many plant defense genes are induced by biotic and abiotic stresses. Salicylic acid (SA), Jasmonic acid (JA), and H_2O_2 have been implicated in playing a central role of plant disease resistance and systemic acquired resistance. Oxalic acid (OA), a compound produced by *Sclerotinia* and many other fungal pathogens *in planta,* plays an important role in the disease infection process.

The expression of LOX, rhoGAP, SCIP-1, and ADH mRNA in the presence of compounds known to induce either systemic acquired resistance or disease response was determined. Six-week-old sunflower leaves were sprayed until runoff with 5 mM SA, 45 μM JA, 5 mM of oxalic acid, and 5 mM H_2O_2. Leaf samples from chemical treated plants were collected at 0, 6, 12, and 24 hours after foliar application.

Northern analysis indicated that there was a significant increase in the steady-state levels of ADH mRNA in SA and H_2O_2 treated leaves (data not shown). The highest level of ADH mRNA expression was detected at 6 hours after application of H_2O_2 and 12 hours following the administration of SA. There was about a 2-to 3-fold increase in rhoGAP transcripts in response to both the H_2O_2 and OA application (data not shown). The mRNA levels of SCIP-1 increased upon treatment with OA.

JA, a product of the LOX pathway, significantly induced the steady-state levels of LOX mRNA. Therefore, LOX mRNA seems to be controlled by a positive feedback loop. In contrast, foliar application of SA and H_2O_2 repressed the expression of LOX mRNA in sunflower. Northern and RNA profiling results revealed that sunflower LOX mRNA was elevated by *Sclerotinia* infection and oxalic acid, a pathogenic factor produced by the fungus (data not shown).

The effects of *Sclerotinia* infection and oxox on SCIP-1, rhoGAP, and ADH mRNA levels was also determined. RNA levels in *Sclerotinia*-infected sunflower and oxox-transgenic sunflower plants were determined using Northern analysis. RNA was isolated from leaves from 6-week-old non-transformed SMF3 plants and from 6-week-old oxox-transgenic plants. Steady state levels of SCIP-1 mRNA significantly increased in 6-week-old leaf tissue from the oxox-transgenic plants as compared to the control

SMF3 leaf samples. RNA was also isolated from stem tissue from 6-week-old non-transformed SMF3 plants and from 6-week-old oxox-transgenic plants. An increase in steady state levels of SCIP-1 mRNA was not detected in the stem tissue (data not shown).

RNA was also isolated from 6-week-old SMF3 plants that were infected 5-days prior to sample collection with *Sclerotinia*. The steady state levels of SCIP-1 RNA following *Sclerotinia* infection increased significantly when compared to RNA levels from uninfected leaf tissue. No change in SCIP-1 RNA levels was seen in the stem tissue following *Sclerotinia* infection (data not shown).

Induction of SCIP-1, ADH, and rhoGAP expression in oxox-transgenic sunflower leaf and stem tissue during development was analyzed. RNA was isolated from leaf and stem tissue from SMF3 plants and oxox-transgenic sunflower plants at 4-week-old, 6-week-old, and 8-week-old stages. Northern blot analysis using RNA samples from SMF3 sunflower plants and samples from oxox-transgenic plants demonstrated SCIP-1 RNA levels increased in leaf tissues in the oxox-transgenic sunflowers. The increase in SCIP-1 RNA levels was most significant at the 8-week-old stage. No detectable induction of SCIP-1 was seen in the stem tissue.

The steady-state-level of ADH mRNA is much higher in stem than in leaf tissue. Adh expression was induced in 8-week-old oxox-transgenic leaf tissue. However, its expression was repressed in oxox-stem tissue. CuraGen QEA assay results indicate that Adh expression was induced by *Sclerotinia* infection in stem and leaf tissues (data not shown).

In the leaf tissue, rhoGAP mRNA level was induced by oxox expression at the 4-week-old stage, and then was repressed with development. In stem tissue, rhoGAP expression was slightly induced by oxox (data not shown).

LOX expression in response to wounding and the ABA signal was determined. Six-week-old sunflower plants (SMF3) were sprayed with 100 μM ABA until the chemical solution started to run off the leaves. For the wounding experiment, each wounded leaf was crushed by a hemostat 20 times and three leaves were treated from each plant. At each time point, six leaves were collected from two treated plants and immediately frozen in liquid N_2. Total RNA was isolated and Northern analysis was performed. Northern blot analysis indicated that wounding significantly induced the

steady state levels of LOX mRNA. The peak of the LOX mRNA accumulation was detected at 6 hours after wounding and high levels of LOX expression was maintained through 72 hours after initial treatment. ABA treatment showed only a slight induction of LOX expression at 12 hours after treatment (data not shown).

Example 3: Transformation and Regeneration of Maize Transgenic Plants

The nucleotide sequences of the present invention can be used to transform sunflower, maize, or other plants using *Agrobacterium* or particle-gun methods. Examples 3, 4, and 5 provide methods for sunflower, maize, and soybean transformations.

Immature maize embryos from greenhouse donor plants are bombarded with a plasmid containing a rhoGAP, LOX, ADH or SCIP-1 nucleotide sequence operably linked to a ubiquitin promoter (Figure 1). The plasmids also contain the selectable marker gene PAT (Wohlleben *et al.* (1988) *Gene 70*:25-37) that confers resistance to the herbicide Bialaphos. Transformation is performed as follows. All media recipes are given in the Appendix. Alternatively, the plant can be transformed with a plasmid comprising the LOX or SCIP-1 promoter sequences of the invention operably linked to the nucleotide sequence encoding the GUS reporter protein (Figure 2).

Preparation of Target Tissue

The ears are surface sterilized in 30% Chlorox bleach plus 0.5% Micro detergent for 20 minutes, and rinsed two times with sterile water. The immature embryos are excised and placed embryo axis side down (scutellum side up), 25 embryos per plate, on 560Y medium for 4 hours and then aligned within the 2.5-cm target zone in preparation for bombardment.

Preparation of DNA

A plasmid vector shown in Figures 1 or 2 is precipitated onto 1.1 μm (average diameter) tungsten pellets using a $CaCl_2$ precipitation procedure as follows:

100 μl prepared tungsten particles in water

10 μl (1 μg) DNA in TrisEDTA buffer (1 μg total)

100 µl 2.5 M $CaCl_2$

10 µl 0.1 M spermidine

Each reagent is added sequentially to the tungsten particle suspension, while maintained on the multitube vortexer. The final mixture is sonicated briefly and allowed to incubate under constant vortexing for 10 minutes. After the precipitation period, the tubes are centrifuged briefly, liquid removed, washed with 500 ml 100% ethanol, and centrifuged for 30 seconds. Again the liquid is removed, and 105 µl 100% ethanol is added to the final tungsten particle pellet. For particle gun bombardment, the tungsten/DNA particles are briefly sonicated and 10 µl spotted onto the center of each macrocarrier and allowed to dry about 2 minutes before bombardment.

Particle Gun Treatment

The sample plates are bombarded at level #4 in particle gun #HE34-1 or #HE34-2. All samples receive a single shot at 650 PSI, with a total of ten aliquots taken from each tube of prepared particles/DNA.

Subsequent Treatment

Following bombardment, the embryos are kept on 560Y medium for 2 days, then transferred to 560R selection medium containing 3 mg/liter Bialaphos, and subcultured every 2 weeks. After approximately 10 weeks of selection, selection-resistant callus clones are transferred to 288J medium to initiate plant regeneration. Following somatic embryo maturation (2-4 weeks), well-developed somatic embryos are transferred to medium for germination and transferred to the lighted culture room. Approximately 7-10 days later, developing plantlets are transferred to 272V hormone-free medium in tubes for 7-10 days until plantlets are well established. Plants are then transferred to inserts in flats (equivalent to 2.5" pot) containing potting soil and grown for 1 week in a growth chamber, subsequently grown an additional 1-2 weeks in the greenhouse, then transferred to classic 600 pots (1.6 gallon) and grown to maturity.

Plants transformed with the plasmid shown in Figure 1 comprising the rhoGAP, LOX, ADH, or SCIP-1 nucleotide sequences are monitored and scored for altered defense response, or altered rhoGAP, LOX, ADH, or SCIP-1 activity.

Plants transformed with the plasmid shown in Figure 2 comprising the LOX or SCIP-1 promoter sequences operably linked to the GUS reporter sequences are monitored for LOX or SCIP-1 promoter activity. Following exposure to various stimuli, such as for example, *Sclerotinia* and oxalic acid, LOX promoter activity is measured using the reporter gene GUS. GUS activity in various tissues is measured by a fluorogenic assay. The fluorogenic assay determines the specific activity of β-glucuronidase (GUS) in various maize tissue extracts. The specific activity of the enzyme is expressed as moles of 4-methyl umbelliferone (MU) released/μg protein/hour. MU is produced when the enzyme (GUS) in plant cell extracts cleaves the glucuronide moiety from the 4-methyl umbelliferyl-β-D-glucuronide (MUG) substrate.

Bombardment and Culture Media

Bombardment medium (560Y) comprises 4.0 g/l N6 basal salts (SIGMA C-1416), 1.0 ml/l Eriksson's Vitamin Mix (1000X SIGMA-1511), 0.5 mg/l thiamine HCl, 120.0 g/l sucrose, 1.0 mg/l 2,4-D, and 2.88 g/l L-proline (brought to volume with D-I H_20 following adjustment to pH 5.8 with KOH); 2.0 g/l Gelrite (added after bringing to volume with D-I H_20); and 8.5 mg/l silver nitrate (added after sterilizing the medium and cooling to room temperature). Selection medium (560R) comprises 4.0 g/l N6 basal salts (SIGMA C-1416), 1.0 ml/l Eriksson's Vitamin Mix (1000X SIGMA-1511), 0.5 mg/l thiamine HCl, 30.0 g/l sucrose, and 2.0 mg/l 2,4-D (brought to volume with D-I H_20 following adjustment to pH 5.8 with KOH); 3.0 g/l Gelrite (added after bringing to volume with D-I H_20); and 0.85 mg/l silver nitrate and 3.0 mg/l bialaphos(both added after sterilizing the medium and cooling to room temperature).

Plant regeneration medium (288J) comprises 4.3 g/l MS salts (GIBCO 11117-074), 5.0 ml/l MS vitamins stock solution (0.100 g nicotinic acid, 0.02 g/l thiamine HCL, 0.10 g/l pyridoxine HCL, and 0.40 g/l glycine brought to volume with polished D-I H_20) (Murashige and Skoog (1962) *Physiol. Plant. 15*:473), 100 mg/l myo-inositol, 0.5 mg/l zeatin, 60 g/l sucrose, and 1.0 ml/l of 0.1 mM abscisic acid (brought to volume with polished D-I H_20 after adjusting to pH 5.6); 3.0 g/l Gelrite (added after bringing to volume with D-I H_20); and 1.0 mg/l indoleacetic acid and 3.0 mg/l bialaphos (added after sterilizing the medium and cooling to 60°C). Hormone-free medium (272V) comprises

4.3 g/l MS salts (GIBCO 11117-074), 5.0 ml/l MS vitamins stock solution (0.100 g/l nicotinic acid, 0.02 g/l thiamine HCL, 0.10 g/l pyridoxine HCL, and 0.40 g/l glycine brought to volume with polished D-I H_20), 0.1 g/l myo-inositol, and 40.0 g/l sucrose (brought to volume with polished D-I H_20 after adjusting pH to 5.6); and 6 g/l bacto-agar (added after bringing to volume with polished D-I H_20), sterilized and cooled to 60° C.

Example 4: *Agrobacterium*-mediated Transformation

For *Agrobacterium*-mediated transformation of maize with rhoGAP, LOX, ADH, or SCIP-1 nucleotide sequences of the invention or a nucleotide sequence operably linked to the LOX or SCIP-1 promoter sequence of the invention, preferably the method of Zhao is employed (U.S. Patent No. 5,981,840, and PCT patent publication WO98/32326; the contents of which are hereby incorporated by reference). Briefly, immature embryos are isolated from maize and the embryos contacted with a suspension of *Agrobacterium*, where the bacteria are capable of transferring the DNA constructs of interest to at least one cell of at least one of the immature embryos (step 1: the infection step). In this step the immature embryos are preferably immersed in an *Agrobacterium* suspension for the initiation of inoculation. The embryos are co-cultured for a time with the *Agrobacterium* (step 2: the co-cultivation step). Preferably the immature embryos are cultured on solid medium following the infection step. Following this co-cultivation period an optional "resting" step is contemplated. In this resting step, the embryos are incubated in the presence of at least one antibiotic known to inhibit the growth of *Agrobacterium* without the addition of a selective agent for plant transformants (step 3: resting step). Preferably the immature embryos are cultured on solid medium with antibiotic, but without a selecting agent, for elimination of *Agrobacterium* and for a resting phase for the infected cells. Next, inoculated embryos are cultured on medium containing a selective agent and growing transformed callus is recovered (step 4: the selection step). Preferably, the immature embryos are cultured on solid medium with a selective agent resulting in the selective growth of transformed cells. The callus is then regenerated into plants (step 5: the regeneration step), and preferably calli grown on selective medium are cultured on solid medium to regenerate the plants.

Example 5: Transformation and Regeneration of Sunflower Plants

The intact meristem method is used for transformation of sunflower plants and expression of the LOX, ADH, rhoGAP, or SCIP-1 nucleotide sequences as follows. Alternatively, the same method could be used to express a nucleotide sequence of interest under the control of the LOX or SCIP-1 promoter sequence of the invention.

Explant Preparation

Seeds are dehulled and surface-sterilized for 20 minutes in a 20% Chlorox™ bleach solution with the addition of two to three drops of Tween 20 per 100 ml of solution, and then rinsed three times with distilled water. Sterilized seeds are imbibed in the dark at 26°C for 20 hours on filter paper moistened with water. Meristem explants are created by removing cotyledons and root radicle from imbibed seeds, and then culturing overnight at 26°C in the dark on 374E medium (1X MS salts, Shepards vitamins, 40 mg/l adenine sulfate, 30 g/l sucrose, o.5 mg/l BAP, 0.25 mg/l IAA, 0.1 mg/l IAA, pH 5.6, 8g/l phytagar). Primary leaves are then removed and explants are transferred to 374M medium (374E except 12 g/l phytagar), arranged in a manner suitable for particle gun bombardment, and cultured overnight at 26°C in the dark.

Preparation of DNA

A plasmid vector comprising the rhoGAP, LOX, ADH, or SCIP-1 nucletoide sequences operably linked to a ubiquitin promoter is constructed (Figure 1). Alternatively, a plasmid vector comprising the LOX promoter or the SCIP-1 nucleotide sequence operably linked to the nucleotide sequence encoding the GUS reporter protein is constructed (Figure 1). Both of these plasmids contain a kanamycin selectable marker gene. The transformation is performed as follows.

Transformation

Approximately 18.8 mg of 1.8 μm tungsten particles are suspended in 150 μl absolute ethanol, and sonicated for 2-4 seconds. After sonication, 10 μl of the suspension is dropped on the center of the surface of a macrocarrier. Each plate of meristem

explants is bombarded twice with 650 psi rupture discs in the top shelf at 26 mm of Hg helium gun vacuum, using a BioRad helium gun.

The plasmid vector shown in Figure 1 or in Figure 2 is introduced into *Agrobacterium* strain EHA 105 (see above) via freeze-thawing as described by Holsters *et al.* (1978) *Mol. Gen. Genet.* 163:181-187. Actively growing, transformed *Agrobacteria* were maintained in shaking liquid cultures using 60A medium with kanamycin (YEP, 50 mg/l kanamycin: 10 g/l yeast extract, 10 g/l bactopeptone, 5 g/l NaCl, pH 7.0, 50 mg/l kanamycin). On the day before the *Agrobacterium* strain is to be used, new liquid cultures are initiated in 60A with kanamycin from the active maintenance culture. They are cultured with shaking at 26°C until they reach an optical density (OD vis = 600 nm) of about 1.0. When the cultures have established this density, they are centrifuged (6000 rpm, 5 min), the supernatant is discarded, and the pellet of bacteria is resuspended in inoculation medium (12.5mM 2-(N-morpholino) ethanesulfonic acid, 1 g/l NH4C1, and 0.3 g/l MgSO4, at pH 5.7), to a final calculated concentration of *Agrobacteria* of 4.0 at OD 600. The particle bombarded explants are inoculated with *Agrobacterium* by first spreading the explants apart on the 374M medium, then placing a droplet of the above suspension directly onto the top of each meristem. The explants are co-cultivated on the medium for 4 days, after which the explants are transferred to 374 C medium (GBA with 1% sucrose and with no BAP, IAA, or GA3, and supplemented with 250 µg/ml cefotaxime). The explants are cultured on this medium for about 2 weeks under 16 hours of daylight, at 26°C.

Recovering Nodes and Plants

Following the 4 days of co-cultivation time on 374M medium, the explants are transferred to 374D (374C medium with 50 mg/l kanamycin) selection medium containing kanamycin. After 2 weeks of selection, explants with associated shoots are transferred to 374C medium and selection resistant shoots are screened using NPTII ELISA. Positive shoots are removed for recovery by in vitro grafting and transformation verified by further NPTII ELISA analysis. Negative shoots are discarded. Explants with smaller shoots which could not be assayed following the 2 weeks on 374D are transferred to 374G (374E with 250 mg/l cefotaxime) for 3-4 days then back to 374C for 2 additional

weeks. Assays are then done to identify positive shoots which are too small to sample in the first round and recovery initiated. Recovered positive shoots are grafted to Pioneer sunflower hybrid *in vitro*-grown sunflower seedling rootstock. The seeds are dehulled and surface-sterilized for 20 minutes in a 20% Chlorox™ bleach solution with two to three drops of Tween20 per 100 ml total volume, and rinsed three times with distilled water. The sterilized seeds are germinated for three days on filter paper moistened with water, then transferred into "48 Medium" (one-half strength MS salts, 0.5% sucrose, 0.3% gelrite, at pH 5.0) and grown at 26°C at 26 in the dark for 3 days, then incubated at 16 hour day culture conditions. The upper portions of selected seedlings are removed, a vertical slice is made in each hypocotyl, and a transformed shoot is inserted into the vertical slice. The cut area is wrapped with parafilm, and after one week culture on the medium, the grafted plants are transferred to soil. In the first two weeks they are maintained under high humidity conditions to acclimatize to the greenhouse environment.

Transformed sectors of TO plants are identified by additional NPTII assays of the greenhouse established positive grafted shoots. After assay, non-transformed sectors are trimmed off to promote auxillary bud development and auxiliary buds from transgenic sectors are recovered so as to establish the best probability to encompass the sector of transformation in germ line cells so that the transformation event is recovered in the next generation. Seed from TO plants are collected, de-hulled, surface sterilized, and germinated on filter paper wetted with water. T1 seedlings are then sampled for NPTII ELISA by removing green cotyledon pieces followed by transfer to seedling growth medium 48P (0.1X MS salts, 0.5% sucrose, pH 5.6, 0.3% gelrite). NPTII positive, actively growing T1 seedlings are transferred at the two leaf stage to soil for growth in the greenhouse. Seed from the confirmed T1 transgenics is used to grow T2 plants.

T2 seeds are planted in a greenhouse. Positive plants are screened by NPTII assay. Various plant tissues are harvested at 80-day-old stage after planting. The harvested material is put in mini-tubes, frozen and stored at –80°C.

Plants transformed with the plasmid shown in Figure 1 comprising the rhoGAP, LOX, ADH, or SCIP-1 nucleotide sequences are monitored and scored for an altered defense response, or a modulation in rhoGAP, LOX, ADH or SCIP-1 activity.

Plants transformed with the plasmid shown in Figure 2 comprising the LOX promoter sequences operably linked to the GUS reporter sequences are monitored for LOX promoter activity. Following exposure to various stimuli that induce the LOX promoter, LOX promoter activity is measured by assaying for GUS activity. GUS activity in various tissues is measured by a fluorogenic assay. The fluorogenic assay determines the specific activity of β-glucuronidase (GUS) in various sunflower tissue extracts. The specific activity of the enzyme is expressed as moles of 4-methyl umbelliferone (MU) released/ug protein/hour. MU is produced when the enzyme (GUS) in plant cell extracts cleaves the glucuronide moiety from the 4-methyl umbelliferyl-β-D-glucuronide (MUG) substrate.

Harvested T2 tissue samples stored at –80°C are homogenized in 400 µl lysis buffer (40 mM Phosphate, pH 7.0, 10 mM EDTA, 10 mM β-mercaptoethanol), and then centrifuged in the Jouan GR422 centrifuge for 10 minutes at 4000 rpm. The total protein concentration of the supernatant is measured using the Bio-Rad Bradford Method (Bio-RAD) with BSA as the standard protein according to manufacture's protocol. Ten µl of diluted supernatant (about 4 µg of total protein) is used for the GUS activity assay. GUS activity is assayed according to Jefferson *et al.* (1987) *EMBO J. 6:* 3901-3907 using MUG as substrate.

As an alternative to the intact meristem method, the split embryonic axis method may be used as described in Malone-Schoneberg *et al.* (1994) *Plant Science 103*:193-207, in transforming sunflower plants with either the plasmid shown in Figure 3 or Figure 4 and generating T2 plants. T2 seeds are planted in a greenhouse and positive plants are screened by NPTII assay. Plant tissues are harvested at 80-day-old stage after planting. The harvested material is put in mini-tubes, frozen, and stored at -80°C.

Example 6: Soybean Embryo Transformation

Soybean embryos are bombarded with a plasmid containing the SCIP-1, rhoGAP, LOX, or ADH sequences operably linked to a ubiquitin promoter (Figure 1) as follows. Alternatively, the soybean embryos can be bombarded with a DNA construct containing the SCIP-1 or LOX promoter operably linked to a nucleotide sequence of interest (Figure

2). To induce somatic embryos, cotyledons, 3-5 mm in length dissected from surface-sterilized, immature seeds of the soybean cultivar A2872, are cultured in the light or dark at 26°C on an appropriate agar medium for six to ten weeks. Somatic embryos producing secondary embryos are then excised and placed into a suitable liquid medium. After repeated selection for clusters of somatic embryos that multiplied as early, globular-staged embryos, the suspensions are maintained as described below.

Soybean embryogenic suspension cultures can maintained in 35 ml liquid media on a rotary shaker, 150 rpm, at 26°C with florescent lights on a 16:8 hour day/night schedule. Cultures are subcultured every two weeks by inoculating approximately 35 mg of tissue into 35 ml of liquid medium.

Soybean embryogenic suspension cultures may then be transformed by the method of particle gun bombardment (Klein *et al.* (1987) *Nature* (London) *327*:70-73, U.S. Patent No. 4,945,050). A Du Pont Biolistic PDS1000/HE instrument (helium retrofit) can be used for these transformations.

A selectable marker gene that can be used to facilitate soybean transformation is a transgene composed of the 35S promoter from Cauliflower Mosaic Virus (Odell *et al.* (1985) *Nature 313*:810-812), the hygromycin phosphotransferase gene from plasmid pJR225 (from *E. coli*; Gritz *et al.* (1983) *Gene 25*:179-188), and the 3' region of the nopaline synthase gene from the T-DNA of the Ti plasmid of *Agrobacterium tumefaciens*. The expression cassette comprising the DNA construct can be isolated as a restriction fragment. This fragment can then be inserted into a unique restriction site of the vector carrying the marker gene.

To 50 µl of a 60 mg/ml 1 µm gold particle suspension is added (in order): 5 µl DNA (1 µg/µl), 20 µl spermidine (0.1 M), and 50 µl $CaCl_2$ (2.5 M). The particle preparation is then agitated for three minutes, spun in a microfuge for 10 seconds and the supernatant removed. The DNA-coated particles are then washed once in 400 µl 70% ethanol and resuspended in 40 µl of anhydrous ethanol. The DNA/particle suspension can be sonicated three times for one second each. Five microliters of the DNA-coated gold particles are then loaded on each macro carrier disk.

Approximately 300-400 mg of a two-week-old suspension culture is placed in an empty 60x15 mm petri dish and the residual liquid removed from the tissue with a

pipette. For each transformation experiment, approximately 5-10 plates of tissue are normally bombarded. Membrane rupture pressure is set at 1100 psi, and the chamber is evacuated to a vacuum of 28 inches mercury. The tissue is placed approximately 3.5 inches away from the retaining screen and bombarded three times. Following bombardment, the tissue can be divided in half and placed back into liquid and cultured as described above.

Five to seven days post bombardment, the liquid media may be exchanged with fresh media, and eleven to twelve days post-bombardment with fresh media containing 50 mg/ml hygromycin. This selective media can be refreshed weekly. Seven to eight weeks post-bombardment, green, transformed tissue may be observed growing from untransformed, necrotic embryogenic clusters. Isolated green tissue is removed and inoculated into individual flasks to generate new, clonally propagated, transformed embryogenic suspension cultures. Each new line may be treated as an independent transformation event. These suspensions can then be subcultured and maintained as clusters of immature embryos or regenerated into whole plants by maturation and germination of individual somatic embryos.

All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.